



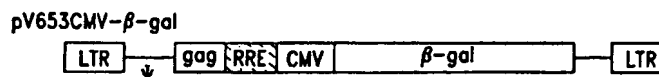
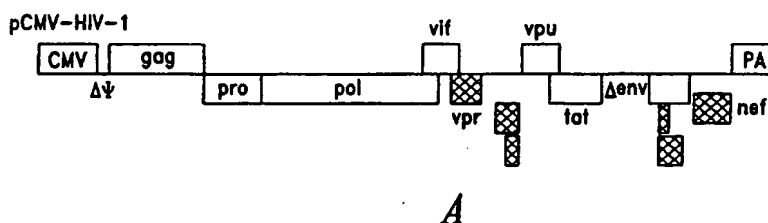
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification:</b> <b>C12N 15/86, 5/10, 7/01, A61K 48/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 99/04026</b> <b>(43) International Publication Date:</b> 28 January 1999 (28.01.99)
<b>(21) International Application Number:</b> PCT/US98/14996 <b>(22) International Filing Date:</b> 20 July 1998 (20.07.98)  <b>(30) Priority Data:</b> 60/053,066      18 July 1997 (18.07.97)      US  <b>(71) Applicant:</b> CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US).  <b>(72) Inventors:</b> CHEN, Shin-Tai; 9995 Red Court, San Diego, CA 92131 (US). GASMI, Mehdi; 3924 8th Avenue, San Diego, CA 92103 (US). YEE, Jiing Kuan; 13951 Durango Drive, Del Mar, CA 92014 (US). JOLLY, Douglas, J.; 277 Hillcrest Drive, Leacadia, CA 92024 (US).  <b>(74) Agents:</b> MCMASTERS, David, D. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>

(54) Title: LENTIVIRAL VECTORS

## (57) Abstract

Lentiviral vectors are provided comprising a 5' lentiviral LTR, a tRNA binding site, a packaging signal, a promoter operably linked to one or more genes of interest, an origin of second strand DNA synthesis and a 3' lentiviral LTR, wherein the lentiviral vector contains a nuclear transport element that is not RRE. Also provided are expression cassettes and packaging cell lines for producing lentiviral vector particles.



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## LENTIVIRAL VECTORS

## TECHNICAL FIELD

The present invention relates generally to pharmaceutical compositions and methods, and more particularly, to lentiviral vectors which are suitable for a wide variety of gene therapy applications.

## BACKGROUND OF THE INVENTION

Since the discovery of nucleic acids in the 1940's and continuing through the most recent era of biotechnology, substantial research has been undertaken in order to realize the possibility that the course of disease may be affected through interaction with the nucleic acids of living organisms. Most recently, a wide variety of methods have been described for altering or affecting genes, including for example, viral vectors derived from retroviruses, adenoviruses, vaccinia viruses, herpes viruses, and adeno-associated viruses (see Jolly, *Cancer Gene Therapy* 1(1):51-64, 1994).

Of these techniques, recombinant retroviral gene delivery methods have been most extensively utilized, in part due to: (1) the efficient entry of genetic material (the vector genome) into cells; (2) an active, efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) the potential to target particular cellular subtypes through control of the vector-target cell binding and the tissue-specific control of gene expression; (5) a general lack of pre-existing host immunity; and (6) substantial knowledge and clinical experience which has been gained with such vectors.

Briefly, retroviruses are diploid positive-strand RNA viruses that replicate through an integrated DNA intermediate. In particular, upon infection by the RNA virus, the retroviral genome is reverse-transcribed into DNA by a virally encoded reverse transcriptase that is carried as a protein in each retrovirus. The viral DNA is then integrated pseudo-randomly into the host cell genome of the infecting cell, forming a "provirus" which is inherited by daughter cells.

One type of retrovirus, the murine leukemia virus, or "MLV", has been widely utilized for gene therapy applications (see generally Mann et al. (*Cell* 33:153, 1983), Cane and Mulligan (*Proc. Nat'l. Acad. Sci. USA* 81:6349, 1984), and Miller et al., *Human Gene Therapy* 1:5-14, 1990). One major disadvantage of MLV-based vectors, however, is that the host range (*i.e.*, cells infected with the vector) is limited, and the frequency of transduction of non-replicating cells is generally low.

In order to improve lentiviral-based gene therapy vectors for certain applications, the present invention provides improved lentiviral based vectors and packaging cell lines. The invention also provides other, related, advantages.

#### SUMMARY OF THE INVENTION

5 Briefly stated, within one aspect of the present invention lentiviral vectors are provided, comprising a 5' lentiviral LTR, a tRNA binding site, a packaging signal, a promoter operably linked to one or more genes of interest, an origin of second strand DNA synthesis and a 3' lentiviral LTR, wherein the lentiviral vector contains a nuclear transport element. The nuclear transport element may be located either  
10 upstream (5') or downstream (3') of the gene of interest. Within certain embodiments, the nuclear transport element is not RRE. Within one embodiment the packaging signal is an extended packaging signal. Within other embodiments the promoter is a tissue specific promoter, or, alternatively, a promoter such as CMV. Within further embodiments, the lentiviral vector further comprises an internal ribosome entry site.

15 Within various embodiments, the lentiviral vector expresses a gene of interest (*e.g.*, a heterologous sequence, although, within certain embodiments lentivirus sequences such as HIV env may also be expressed). Representative examples of suitable genes of interest include cytokines, insulin,  $\beta$ -gal, alkaline phosphatase (*e.g.*, placental alkaline phosphatase), green fluorescence protein, factor VIII, factor IX, LDL  
20 receptor, human growth hormone, EPO, TPO, prodrug activating enzymes, trans-dominant negative viral or cancer-associated proteins, and tyrosine hydroxylase.

A wide variety of lentiviruses may be utilized within the context of the present invention, including for example, lentiviruses selected from the group consisting of HIV, HIV-1, HIV-2 and SIV.

25 Within other aspects of the invention expression cassettes are provided. Within one embodiment, gag/pol expression cassettes are provided comprising a promoter and a sequence encoding gag/pol and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to gag/pol and vpr, vpu, nef or vif. Within another embodiment, tat expression cassettes are provided comprising a promoter and a  
30 sequence encoding tat and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to tat and vpr, vpu, nef or vif. Within further embodiments rev expression cassettes are provided comprising a promoter and a sequence encoding rev and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to rev and vpr, vpu, nef or vif. Within yet other embodiments, VSV-G expression cassettes  
35 are provided comprising a promoter and a sequence encoding VSV-G and at least one

of vpr, vpu, nef or vif, wherein the promoter is operably linked to VSV-G and vpr, vpu, nef or vif.

Within yet other aspects of the invention, host cells (*e.g.*, packaging cell lines) are provided within contain any of the expression cassettes described herein. For example, within one aspect packaging cell line are provided comprising an expression cassette that comprises a sequence encoding gag/pol, and a nuclear transport element, wherein the promoter is operably linked to the sequence encoding gag/pol. Within other aspects, packaging cell lines are provided comprising a promoter and a sequence encoding tat, rev, or an envelope (*e.g.*, VSV-G), wherein the promoter is operably linked to the sequence encoding tat, rev, or, the envelope. Within further embodiments, the packaging cell line may further comprise a sequence encoding any one or more of nef, vif, vpu or vpr. Within another embodiment, the expression cassette is stably integrated. Within yet another embodiment, the packaging cell line, upon introduction of a lentiviral vector, produces particles at a concentration of greater than  $10^5$  cfu/ml. Within further embodiments the promoter is inducible. Within certain preferred embodiments of the invention, the packaging cell line, upon introduction of a lentiviral vector, produces particles that are free of replication competent virus.

Within yet other aspects of the present invention, packaging cell lines are provided comprising an expression cassette which directs the expression of a *gag/pol* gene, an expression cassette which directs the expression of an *env* gene (*e.g.*, VSV-G, or an amphotrophic envelope), an expression cassette which directs the expression of Tat, and expression cassette which directs the expression of Rev. Within further aspects, a lentiviral vector is introduced into the packaging cell line to produce a vector producing cell line.

Within further aspects, methods are provided for enhancing production of infectious virus, comprising the step of infecting packaging cell lines with a viral vector, wherein a butyrate salt (*e.g.*, sodium butyrate or potassium butyrate) is added subsequent to, or after infection of the packaging cell line. Within certain embodiments, the butyrate salt is added prior to the step of harvesting infectious virus.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of one representative HIV-1 based vector.

Figures 2A, 2B and 2C are schematic illustrations of pCMV-HIV-1, pV653CMV- $\beta$ gal, and pCMU-G, respectively.

Figures 3A, 3B, 3C and 3D are schematic illustrations of pCH-GP-1, pCH-GP-2, pCH-GP-3 and pCH-GP-4, respectively.

Figures 4A and 4B are schematic illustrations of pCMV-tat and pTetO-rev.

Figure 5 is a flow chart of one representative method for packaging cell line generation.

Figure 6 is a schematic illustration which shows a comparison of the genome organizations of lenti- and oncoretroviruses.

Figure 7 is a table which shows the affected HIV-1 accessory proteins on vector production.

Figure 8 is a table which shows the expression level of p24 in pCHGP transfected cells.

Figure 9 is a bar graph which shows stimulation of vector production by sodium butyrate. Briefly, vectors derived from either pCMV-HIV-1 (closed boxes) or PCHGP-2 (striped boxes) were generated in 293T cells in the presence of various concentrations of sodium butyrate as indicated. Titers were determined in HT1080 cells as described in materials and methods. Values are the ratios of titers with sodium butyrate over titers without sodium butyrate for each vector.

Figure 10 is a bar graph which shows transduction efficiency in HeLa cells. Briefly, 300  $\mu$ l of different viral preparations were used to transduce actively-dividing or growth-arrested HeLa cells in 12 well plates. The cells were harvested two days after transduction and the total  $\beta$ -galactosidase activity was determined by blue cell count after X-Gal staining. The results are presented as a percentage value of vector titer observed in HT1080 cells for each viral preparation ([Titer in HeLa (dividing or quiescent)/Titer in HT1080] X 100).

Figure 11 is a bar graph which shows the transduction efficiency in human skin fibroblasts. Briefly, 10  $\mu$ l of different types were used to infect dividing and quiescent fibroblast in a 12 well plate. Two days after transduction, titer was determined by blue cell counting after X-Gal staining. Data for transduction of quiescent and dividing fibroblasts, are presented as a percentage value of titer observed

in growing HT1080 for each viral preparation ([Titer in fibroblasts (growing or quiescent)/Titer in HT1080] X 100). The values are an average of four experiments.

#### DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Vector construct", "lentiviral vector", and "recombinant lentiviral vector" refers to a nucleic acid construct which carries, and within certain embodiments, is capable of directing the expression of a nucleic acid molecule of interest. The lentiviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the recombinant lentiviral vector may also include a signal which directs polyadenylation, selectable markers such as Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. By way of example, such vectors typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof.

"Lentiviral vector particle" as utilized within the present invention refers to a lentivirus which carries at least one gene of interest. The retrovirus may also contain a selectable marker. The recombinant lentivirus is capable of reverse transcribing its genetic material into DNA and incorporating this genetic material into a host cell's DNA upon infection. Lentiviral vector particles may have a lentiviral envelope, a non-lentiviral envelope (e.g., an amphi or VSV-G envelope), or a chimeric envelope.

"Nucleic acid expression vector" or "Expression cassette" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The nucleic acid expression vector must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence. Within certain embodiments of the invention, the nucleic acid expression vectors described herein may be contained within a plasmid construct. In addition to the components of the nucleic acid expression vector, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which

allows the plasmid construct to exist as single-stranded DNA (e.g., a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

"Packaging cell" refers to a cell which contains those elements necessary for production of infectious recombinant retrovirus which are lacking in a recombinant retroviral vector. Typically, such packaging cells contain one or more expression cassettes which are capable of expressing proteins which encode *gag*, *pol* and *env* proteins.

"Producer cell" or "Vector producing cell" refers to a cell which contains all elements necessary for production of recombinant retroviral vector particles.

#### CONSTRUCTION AND PREPARATION OF LENTIVIRAL VECTORS

As noted above, the present invention provides lentiviral vectors which are designed to carry or express a selected gene(s) or sequence(s) of interest. Lentiviral vectors may be readily constructed from a wide variety of lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Representative examples of lentiviruses included HIV, HIV-1, HIV-2 and SIV. Such lentiviruses may either be obtained from patient isolates, or, more preferably, from depositories or collections such as the American Type Culture Collection (ATCC, Rockville, MD), or isolated from known sources using commonly available techniques.

Any of the above lentiviruses may be readily utilized in order to assemble or construct lentiviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (e.g., Sambrook *et al*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). In addition, within certain embodiments of the invention, portions of the lentiviral gene delivery vehicles may be derived from different viruses. For example, within one embodiment of the invention, recombinant lentiviral vector LTRs may be derived from an HIV, a packaging signal from SIV, and an origin of second strand synthesis from HIV-2.

Within one aspect of the present invention, lentiviral vector constructs are provided comprising a 5' lentiviral LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein said lentiviral vector contains a nuclear transport element that is not RRE. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter



and enhancer elements which are located within U3. LTRs may be readily identified in the provirus (integrated DNA form) due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred recombinant retroviral vector constructs which are provided herein also comprise a packaging signal, as well as one or more genes of interest, each of which is discussed in more detail below. In addition, the lentiviral vectors have a nuclear transport element which, in preferred embodiments is not RRE. Representative examples of suitable nuclear transport elements include the element in Rous sarcoma virus (Ogert, et al., *J. Virol.* 70, 3834-3843, 1996), the element in Rous sarcoma virus (Liu & Mertz, *Genes & Dev.*, 9, 1766-1789, 1995) and the element in the genome of simian retrovirus type 1 (Zolotukhin, et al., *J. Virol.* 68, 7944-7952, 1994). Other potential elements include the elements in the histone gene (Kedes, *Annu. Rev. Biochem.* 48, 837-870, 1970), the  $\alpha$  interferon gene (Nagata et al., *Nature* 287, 401-408, 1980), the  $\beta$ -adrenergic receptor gene (Koilkka, et al., *Nature* 329, 75-79, 1987), and the c-Jun gene (Hattorie, et al., *Proc. Natl. Acad. Sci. USA* 85, 9148-9152, 1988).

Within one aspect of the invention, recombinant lentiviral vector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the recombinant lentiviral vector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or

*env* expression cassettes that are used to construct packaging cell lines for the recombinant retroviral vector constructs are set forth in more detail below and in Example 1.

As an illustration, within one embodiment of the invention construction of recombinant lentiviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging.

Within certain embodiments of the invention, lentiviral vectors are provided wherein tissue-specific promoters are utilized to drive expression of one or more genes of interest. For example, lentiviral vector particles of the invention can contain a liver specific promoter to maximize the potential for liver specific expression of the exogenous DNA sequence contained in the vectors. Preferred liver specific promoters include the hepatitis B X-gene promoter and the hepatitis B core protein promoter. These liver specific promoters are preferably employed with their respective enhancers. The enhancer element can be linked at either the 5' or the 3' end of the nucleic acid encoding the therapeutic molecule. The hepatitis B X gene promoter and its enhancer can be obtained from the viral genome as a 332 base pair EcoRV-NcoI DNA fragment employing the methods described in Twu, 1987, *J. Virol.* 61:3448-3453. The hepatitis B core protein promoter can be obtained from the viral genome as a 584 base pair BamHI-BglII DNA fragment employing the methods described in Gerlach, 1992, *Virol* 189:59-66. It may be necessary to remove the negative regulatory sequence in the BamHI-BglII fragment prior to inserting it. Other liver specific promoters include the AFP (alpha fetal protein) gene promoter and the albumin gene promoter, as disclosed in EP Patent Publication 0 415 731, the -1 antitrypsin gene promoter, as disclosed in Rettenger, 1994, *Proc. Natl. Acad. Sci.* 91:1460-1464, the fibrinogen gene promoter, the APO-A1 (Apolipoprotein A1) gene promoter, and the promoter genes for liver transference enzymes such as, for example, SGOT, SGPT and -glutamyle transferase. See also PCT Patent Publications WO 90/07936 and WO 91/02805 for a description of the use of liver specific promoters in lentiviral vector particles.

Within certain embodiments of the invention, the lentiviral vector constructs provided herein may be generated such that more than one gene of interest is expressed. This may be accomplished through the use of di- or oligo-cistronic cassettes (e.g., where the coding regions are separated by 80 nucleotides or less, see generally

Levin et al., *Gene* 108:167-174, 1991), or through the use of Internal Ribosome Entry Sites ("IRES").

#### PACKAGING / PRODUCER CELL LINES

Packaging cell lines suitable for use with the above described  
5 recombinant retroviral vector constructs may be readily prepared given the disclosure provided herein. Briefly, the parent cell line from which the packaging cell line is derived can be selected from a wide variety of mammalian cell lines, including for example, human cells, monkey cells, dog cells, mouse cells, and the like.

Within one embodiment of the invention, potential packaging cell line  
10 candidates are screened by isolating the human placental alkaline phosphatase (PLAP) gene from pBAAP, and inserting the gene into pNL4-3. To generate infectious virus, the construct is co-transfected with pCMV-G into 293 cells, and the virus harvested 48 hours after transfection. The resulting virus can be utilized to infect candidate host cells (e.g., human cells such as HeLa, HY1080, 293, Jurkats, supT1 and CEM), which are  
15 subsequently sorted using antibodies specific for PLAP. Production of p24 and reverse transcriptase can also be analyzed in the assessment of suitable packaging cell lines.

After selection of a suitable host cell for the generation of a packaging cell line, one or more expression cassettes are introduced into the cell line in order to  
20 complement or supply in *trans* components of the vector which have been deleted (*see generally* U.S. Serial No. 08/240,030, filed May 9, 1994; *see also* U.S. Serial No. 07/800,921, filed November 27, 1991).

Representative examples of suitable expression cassettes include gag/pol expression cassettes which comprise a promoter and a sequence encoding gag/pol and at  
25 least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to gag/pol and vpr, vpu, nef or vif. Within another embodiment, tat expression cassettes are provided comprising a promoter and a sequence encoding tat and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to tat and vpr, vpu, nef or vif. Within further embodiments rev expression cassettes are provided comprising a promoter and a  
30 sequence encoding rev and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to rev and vpr, vpu, nef or vif. Within yet other embodiments, VSV-G expression cassettes are provided comprising a promoter and a sequence encoding VSV-G and at least one of vpr, vpu, nef or vif, wherein the promoter is operably linked to VSV-G and vpr, vpu, nef or vif.

Utilizing the above-described expression cassettes, a wide variety of packaging cell lines can be generated. For example, within one aspect packaging cell line are provided comprising an expression cassette that comprises a sequence encoding gag/pol, and a nuclear transport element, wherein the promoter is operably linked to the sequence encoding gag/pol. Within other aspects, packaging cell lines are provided comprising a promoter and a sequence encoding tat, rev, or an envelope (*e.g.*, VSV-G), wherein the promoter is operably linked to the sequence encoding tat, rev, or, the envelope. Within further embodiments, the packaging cell line may further comprise a sequence encoding any one or more of nef, vif, vpu or vpr. For example, the packaging cell line may contain only nef, vif, vpu, or vpr alone, nef and vif, nef and vpu, nef and vpr, vif and vpu, vif and vpr, vpu and vpr, nef vif and vpu, nef vif and vpr, nef vpu and vpr, vvir vpu and vpr, or, all four of nef vif vpu and vpr.

Within another embodiment, the expression cassette is stably integrated. Within yet another embodiment, the packaging cell line, upon introduction of a lentiviral vector, produces particles at a concentration of greater than  $10^5$ ,  $10^6$ ,  $10^7$ ,  $10^8$ , or,  $10^9$  cfu/ml. Within further embodiments the promoter is inducible. Within certain preferred embodiments of the invention, the packaging cell line, upon introduction of a lentiviral vector, produces particles that are free of replication competent virus.

#### GENES OF INTEREST / HETEROLOGOUS NUCLEIC ACID MOLECULES

A wide variety of nucleic acid molecules may be carried and/or expressed by the lentiviral vector particles of the present invention. As used herein, "pathogenic agent" refers to a cell that is responsible for a disease state. Representative examples of pathogenic agents include tumor cells, autoreactive immune cells, hormone secreting cells, cells which lack a function that they would normally have, cells that have an additional inappropriate gene expression which does not normally occur in that cell type, and cells infected with bacteria, viruses, or other intracellular parasites. In addition, as used herein "pathogenic agent" may also refer to a cell that has become tumorigenic due to inappropriate insertion of nucleic acid molecules contained by the lentiviral vector into a host cell's genome.

Examples of nucleic acid molecules which may be carried and/or expressed by lentiviral vector particles of the present invention include genes and other nucleic acid molecules which encode a substance, as well as biologically active nucleic acid molecules such as inactivating sequences that incorporate into a specified intracellular nucleic acid molecule and inactivate that molecule. A nucleic acid molecule is considered to be biologically active when the molecule itself provides the

desired benefit. For example, the biologically active nucleic acid molecule may be an inactivating sequence that incorporates into a specified intracellular nucleic acid molecule and inactivates that molecule, or the molecule may be a tRNA, rRNA or mRNA that has a configuration that provides a binding capability.

5 Substances which may be encoded by the nucleic acid molecules described herein include proteins (*e.g.*, antibodies including single chain molecules), immunostimulatory molecules (such as antigens) immunosuppressive molecules, blocking agents, palliatives (such as toxins, antisense ribonucleic acids, ribozymes, enzymes, and other material capable of inhibiting a function of a pathogenic agent)  
10 cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction,  
15 immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amyloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and  
20 pyrimidines.

For palliatives, when "capable of inhibiting a function" is utilized within the context of the present invention, it should be understood that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the  
25 pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for cancerous diseases include cell replication, susceptibility to external signals (*e.g.*, contact inhibition), and lack of production of anti-oncogene proteins. Examples of such functions for cardiovascular  
30 disease include inappropriate growth or accumulation of material in blood vessels, high blood pressure, undesirable blood levels of factors such as cholesterol or low density lipoprotein that predispose to disease, localized hypoxia, and inappropriately high and tissue-damaging levels of free radicals. Examples of such functions for neurological conditions include pain, lack of dopamine production, inability to replace damaged  
35 cells, deficiencies in motor control of physical activity, inappropriately low levels of various peptide hormones derived from neurological tissue such as the pituitary or

hypothalamus, accumulation of Alzheimer's Disease associated amyloid plaque protein, and inability to regenerate damaged nerve junctions. Examples of such functions for autoimmune or inflammatory disease include inappropriate production of cytokines and lymphokines, inappropriate production and existence of autoimmune antibodies and  
5 cellular immune responses, inappropriate disruption of tissues by proteases and collagenases, lack of production of factors normally supplied by destroyed cells, and excessive or aberrant regrowth of tissues under autoimmune attack.

Within one aspect of the present invention, methods are provided for administration of a recombinant lentivirus which directs the expression of a palliative.  
10 Representative examples of palliatives that act directly to inhibit the growth of cells include toxins such as ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J.*  
15 *Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez & Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.* 21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-  
20 528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987). A detailed description of recombinant retroviruses which express Russel's Viper Venom is provided in U.S. Serial No. 08/368,574, filed December 30, 1994.

25 Within other aspects of the invention, the lentiviral vector carries a gene specifying a product which is not in itself toxic, but when processed or modified by a protein, such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, recombinant retrovirus could carry a gene encoding a proprotein chain, which becomes toxic upon processing by the HIV protease. More specifically, a  
30 synthetic inactive proprotein form of the toxic ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

Within a related aspect of the present invention, lentiviral vectors are provided which direct the expression of a gene product(s) that activates a compound  
35 with little or no cytotoxicity into a toxic product. Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no

cytotoxicity into a toxic product may be utilized within the context of the present invention. Representative examples of such gene products include HSVTK and VZVTK which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs ganciclovir, acyclovir, or any of their analogues (e.g., FIAC, DHPG) to HSVTK, phosphorylates the drug into its corresponding active nucleotide triphosphate form.

For example, within one embodiment of the invention, the lentiviral vector directs the expression of the herpes simplex virus thymidine kinase ("HSVTK") gene downstream, and under the transcriptional control of an HIV promoter (which is known to be transcriptionally silent except when activated by HIV tat protein). Briefly, expression of the tat gene product in human cells infected with HIV and carrying the recombinant retrovirus causes increased production of HSVTK. The cells (either *in vitro* or *in vivo*) are then exposed to a drug such as ganciclovir, acyclovir or its analogues (FIAC, DHPG). As noted above, these drugs are known to be phosphorylated by HSVTK (but not by cellular thymidine kinase) to their corresponding active nucleotide triphosphate forms. Acyclovir triphosphates inhibit cellular polymerases in general, leading to the specific destruction of cells expressing HSVTK in transgenic mice (see Borrelli et al., *Proc. Natl. Acad. Sci. USA* 85:7572, 1988). Those cells containing the recombinant retrovirus and expressing HIV tat protein are selectively killed by the presence of a specific dose of these drugs.

In a manner similar to the preceding embodiment, lentiviral vectors may be generated which carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. Such genes may have no equivalent in mammalian cells, and might come from organisms such as a virus, bacterium, fungus, or protozoan. Representative examples include: *E. coli* guanine phosphoribosyl transferase ("gpt") gene product, which converts thioxanthine into thioxanthine monophosphate (see Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (e.g., *Fusarium oxysporum*) or bacterial cytosine deaminase which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS* 89:33, 1992); carboxypeptidase G2 which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxyacetabide derivatives of doxorubicin and melphalan to toxic compounds. Conditionally lethal gene products of

this type have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug, which is not a purine or pyrimidine analogue, to a cytotoxic form (see Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

Additionally, in the instance where the target pathogen is a mammalian virus, lentiviral vectors may be constructed to take advantage of the fact that mammalian viruses in general tend to have "immediate early" genes, which are necessary for subsequent transcriptional activation of other viral promoter elements. Gene products of this nature are excellent candidates for intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes transcribed from transcriptional promoter elements that are responsive to such viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human and interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, from these viral-responsive elements (VREs) could result in the destruction of cells infected with a variety of different viruses.

In another embodiment of the invention, lentiviral vectors are provided that produce substances such as inhibitor palliatives, that inhibit viral assembly. In this context, the recombinant retrovirus codes for defective *gag*, *pol*, *env* or other viral particle proteins or peptides which inhibit in a dominant fashion the assembly of viral particles. Such inhibition occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

One way of increasing the effectiveness of inhibitory palliatives is to express inhibitory genes, such as viral inhibitory genes, in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, a recombinant retrovirus may be administered that inhibits HIV replication (by expressing anti-sense *tat*, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

In another embodiment of the invention, lentiviral vectors are provided for the expression substances such as inhibiting peptides or proteins specific for viral



protease. Viral protease cleaves the viral *gag* and *gag/pol* proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. The HIV protease is known to be an aspartyl protease, and these are known to be inhibited by peptides made from amino acids from protein or analogues. Lentiviral vectors that inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

Administration of the lentiviral vectors discussed above should be effective against many virally linked diseases, cancers, or other pathogenic agents.

In yet another aspect, lentiviral vectors are provided which have a therapeutic effect by encoding one or more ribozymes (RNA enzymes) (Haseloff and Gerlach, *Nature* 334:585, 1989) which will cleave, and hence inactivate, RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA sequence corresponding to a pathogenic state, such as HIV tat, and toxicity is specific to such pathogenic state. Representative examples of suitable ribozymes include hammerhead ribozymes (see Rossi et al., *Pharmac. Ther* 50:245-254, 1991) and hairpin ribozymes (Hämpel et al., *Nucl. Acids Res.* 18:299-304, 1990; U.S. Patent No. 5,254,678) and *Tetrahymena* based ribozymes (U.S. Patent No. 4,987,071). Additional specificity may be achieved in some cases by making this a conditional toxic palliative, as discussed above.

In still another aspect, lentiviral vectors are provided comprising a biologically active nucleic acid molecule that is an antisense sequence (an antisense sequence may also be encoded by a nucleic acid sequence and then produced within a host cell via transcription). Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein, or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway. In other embodiments, the antisense sequence is selected from the group consisting of sequences which encode influenza virus, HIV, HSV, HPV, CMV, and HBV. The antisense sequence may also be an antisense RNA complementary to RNA sequences necessary for pathogenicity.

Alternatively, the biologically active nucleic acid molecule may be a sense RNA (or DNA) complementary to RNA sequences necessary for pathogenicity.

Within a further embodiment of the invention antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

In another embodiment, lentiviral vectors of the invention express a surface protein that is itself therapeutically beneficial. For example, in the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

Still further aspects of the present invention relate to lentiviral vectors capable of immunostimulation. Briefly, the ability to recognize and defend against foreign pathogens is essential to the function of the immune system. In particular, the immune system must be capable of distinguishing "self" from "nonself" (*i.e.*, foreign), so that the defensive mechanisms of the host are directed toward invading entities instead of against host tissues. Cytolytic T lymphocytes (CTLs) are typically induced, or stimulated, by the display of a cell surface recognition structure, such as a processed, pathogen-specific peptide, in conjunction with a MHC class I or class II cell surface protein.

Diseases suitable to treatment include viral infections such as influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II and CMV, cancers such as melanomas, renal carcinoma, breast cancer, ovarian cancer and other cancers, and heart disease.

5 In one embodiment, the invention provides methods for stimulating a specific immune response and/or inhibiting viral spread by using lentiviral vectors that direct the expression of an antigen or modified form thereof in susceptible target cells, wherein the antigen is capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for  
10 viral interactions. Expression of the protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the lentiviral vector is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the  
15 correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogs thereof (*e.g.*, Altmann et al., *Nature* 338:512, 1989).

An immune response can also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) (a) the gene for the specific T-cell  
20 receptor that recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), (b) the gene for an immunoglobulin which recognizes the antigen of interest, or (c) the gene for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, recombinant retroviruses may also be used as an immunostimulant, immunomodulator, or vaccine, etc.

25 In the particular case of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from a recombinant retrovirus may be in a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120, and gp 41, which have been modified to  
30 reduce their pathogenicity. In particular, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but haplotype-specific epitopes or to present several haplotype-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The haplotype-specific epitopes can be  
35 further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or

combinations of genes, such as gag, pol, rev, vif, nef, prot, gag/pol, gag prot, etc., may also provide protection in particular cases.

HIV is only one example. This approach may be utilized for many virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed. Representative examples of such "disease-associated" antigens all or portions of various eukaryotic (including for example, parasites), prokaryotic (e.g., bacterial) or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV"; see U.S. Serial No. 08/102/132), Human Papiloma Virus ("HPV"; see WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV"; see EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV"; see U.S. Serial No. 07/948,358; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV"; U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV"; see U.S. Serial No. 07/965,084).

In accordance with the immunostimulation aspects of the invention, substances which are carried and/or expressed by the lentiviral vectors of the present invention may also include "immunomodulatory factors," many of which are set forth above. Immunomodulatory factors refer to factors that, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The factor may also be expressed from a non-recombinant retrovirus derived gene, but the expression is driven or controlled by the recombinant retrovirus. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (e.g., <sup>3</sup>H thymidine uptake), and *in vitro* cytotoxic assays (e.g., which measure <sup>51</sup>Cr release) (see, Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*.

Representative examples of such factors include cytokines, such as IL-1, IL-2 (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper et al., *Cell* 57:503-512, 1989; Golumbek et al., *Science* 254:713-716, 1991; U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12, IL-13 (*Cytokine Bulletin*, Summer

1994), IL-14 and IL-15, particularly IL-2, IL-4, IL-6, IL-12, and IL-13, alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA* 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872, 1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 2008-2015, 1991; Watanabe et al., *PNAS* 86:9456-9460, 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother.* 30:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman et al., *J. Immunology* 144:942-951, 1990), CD3 (Krissanen et al., *Immunogenetics* 26:258-266, 1987), ICAM-1 (Altman et al., *Nature* 338:512-514, 1989; Simmons et al., *Nature* 331:624-627, 1988), ICAM-2, LFA-1, LFA-3 (Wallner et al., *J. Exp. Med.* 166(4):923-932, 1987), MHC class I molecules, MHC class II molecules, B7.1-.3,  $\beta_2$ -microglobulin (Parnes et al., *PNAS* 78:2253-2257, 1981), chaperones such as calnexin, MHC linked transporter proteins or analogs thereof (Powis et al., *Nature* 354:528-531, 1991). Immunomodulatory factors may also be agonists, antagonists, or ligands for these molecules. For example soluble forms of receptors can often behave as antagonists for these types of factors, as can mutated forms of the factors themselves.

The choice of which immunomodulatory factor to include within a lentiviral vector may be based upon known therapeutic effects of the factor, or, experimentally determined. For example, a known therapeutic effector in chronic hepatitis B infections is alpha interferon. This has been found to be efficacious in compensating a patient's immunological deficit, and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory factor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA matched cells (e.g., EBV transformed cells) that have been transduced with a recombinant retrovirus which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory factor. These stimulated PBLs are then used as effectors in a CTL assay with the HLA matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory factor. Within one embodiment of the invention, the immunomodulatory factor gamma interferon is particularly preferred.

The present invention also includes lentiviral vectors which encode immunogenic portions of desired antigens including, for example, viral, bacterial or parasite antigens. For example, at least one immunogenic portion of a hepatitis B antigen can be incorporated into a lentiviral vector. The immunogenic portion(s) which are incorporated into the lentiviral vector may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long, and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature* 351:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Within one embodiment of the present invention, at least one immunogenic portion of a hepatitis C antigen can be incorporated into a lentiviral vector. Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology* 14:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polypeptide may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict CTL epitopes. For example, CTL epitopes for the HLA A2.1 haplotype may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature* 351:290, 1991). From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Other disease-associated antigens which may be carried by the gene delivery constructs of the present invention include, for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (see U.S. Serial No. 08/104,424). Representative examples of altered cellular components which are normally associated with tumor cells include ras\* (wherein "\*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53\*, Rb\*, altered protein encoded by Wilms' tumor gene, ubiquitin\*, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, Platelet Derived Growth

Factor ("PDGF") receptor, insulin receptor, Epidermal Growth Factor ("EGF") receptor, and the Colony Stimulating Factor ("CSF") receptor.

Immunogenic portions of the disease-associated antigens described herein may be selected by a variety of methods. For example, the HLA A2.1/K<sup>b</sup> transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/K<sup>b</sup> transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Immunogenic proteins of the present invention may also be manipulated by a variety of methods known in the art, in order to render them more immunogenic. Representative examples of such methods include: adding amino acid sequences that correspond to T helper epitopes; promoting cellular uptake by adding hydrophobic residues; by forming particulate structures; or any combination of these (*see generally*, Hart, *op. cit.*, Milich et al., *Proc. Natl. Acad. Sci. USA* 85:1610-1614, 1988; Willis, *Nature* 340:323-324, 1989; Griffiths et al., *J. Virol.* 65:450-456, 1991).

Sequences which encode the above-described nucleic acid molecules may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Other nucleic acid molecules that encode the above-described substances, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha

interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which  
5 contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Molecularly cloned genomes which encode the hepatitis B virus may be  
10 obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the *Bam*H I site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981). (Note that correctable errors occur in  
15 the sequence of ATCC No. 45020.)

Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single  
20 stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159). See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq  
25 polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Nucleic acid molecules which are carried and/or expressed by the lentiviral vectors described herein may also be synthesized, for example, on an Applied  
30 Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, California)).

#### METHODS FOR UTILIZING LENTIVIRUS VECTOR PARTICLES

As noted above, the present invention also provides methods for delivering a selected heterologous sequence to a vertebrate or insect, comprising the  
35 step of administering to a vertebrate or insect a lentiviral vector particle as described



herein which is capable of expressing the selected heterologous sequence. Such lentiviral vector particles may be administered either directly (*e.g.*, intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Fung et al., *Proc. Natl. Acad. Sci. USA* 80:353-357, 1983; Seeger et al., *Proc. Natl. Acad. Sci. USA* 81:5849-5852; Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes of several types (*see, e.g.*, Wang et al., *PNAS* 84:7851-7855, 1987); CaPO<sub>4</sub> (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the lentiviral vector particles may either be administered directly (*i.e., in vivo*), or to cells which have been removed (*ex vivo*), and subsequently returned.

As discussed in more detail below, lentiviral vector particles may be administered to a vertebrate or insect organism or cell for a wide variety of both therapeutic or productive purposes, including for example, for the purpose of stimulating a specific immune response; inhibiting the interaction of an agent with a host cell receptor; to express a toxic palliative, including for example, conditional toxic palliatives; to immunologically regulate the immune system; to express markers, for replacement gene therapy and/or to produce a recombinant protein. These and other uses are discussed in more detail below.

#### 1. Immunostimulation

Within one aspect of the present invention, compositions and methods are provided for administering a lentiviral vector particle which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV, HCV, HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease. More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited.

Representative examples of pathogenic agents include bacteria, fungi, parasites, viruses and cancer cells.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using a lentiviral vector particle that directs the expression of an antigen or modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the lentiviral vector is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (*e.g.*, Altmann et al., *Nature* 338:512, 1989). Cells infected with lentiviral vector particles are expected to do this efficiently because they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, lentiviral vectors can easily be thought of as safe viral vectors which can be used on healthy individuals for vaccine use.

This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into a lentiviral vector particle, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response may also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T cell receptor which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, the lentiviral vector particles may be used as an immunostimulant, immunomodulator, or vaccine.

In another embodiment of the invention, methods are provided for producing inhibitor palliatives wherein lentiviral vector particles deliver and express defective interfering viral structural proteins, which inhibit viral assembly. Such lentiviral vector particles may encode defective *gag*, *pol*, *env* or other viral particle proteins or peptides and these would inhibit in a dominant fashion the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

In another embodiment of the invention, methods are provided for the expression of inhibiting peptides or proteins specific for viral protease. Briefly, viral protease cleaves the viral *gag* and *gag/pol* proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. As an example, the HIV protease is known to be an aspartyl protease and these are known to be inhibited by peptides made from amino acids from protein or analogues. Lentiviral vectors to inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

Another embodiment involves the delivery of suppressor genes which, when deleted, mutated, or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a lentiviral vector particle leads to regression of the tumor phenotype in these cells. Examples of such cancers are retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, administration of the lentiviral vector particle and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In yet another embodiment, the lentiviral vector provides a therapeutic effect by transcribing a ribozyme (an RNA enzyme) (Haseloff and Gerlach, *Nature* 334:585, 1989) which will cleave and hence inactivate RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA species such as a RNA or a retroviral genome.

Additional specificity may be achieved in some cases by making this a conditional toxic palliative (*see below*).

One way of increasing the effectiveness of inhibitory palliatives is to express viral inhibitory genes in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, lentiviral vector particles may be delivered which inhibit HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

## 2. Blocking Agents

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors produced by themselves or other cells. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers or other proliferative conditions (*e.g.*, restenosis), cells may respond inappropriately or not at all to signals from other cells or factors, or specific factors may be mutated, overexpressed, or underexpressed, resulting in loss of appropriate cell cycle control. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by producing, *in vivo*, an analogue to either of the partners in an interaction. Alternatively, cell cycle control may be restored by preventing the transition from one phase to another (*e.g.*, G1 to S phase) using a blocking factor which is absent or underexpressed. This blocking action may occur intracellularly, on the cell membrane, or extracellularly, and the action of the lentivirus vector particle carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a lentiviral vector expressing either an HIV *env* analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-

containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Administration of a lentiviral vector particle encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment  
5 can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

In the case of uncontrolled proliferative states, such as cancer or restenosis, cell cycle progression may be halted by the expression of a number of  
10 different factors that affect signaling by cyclins or cyclin-dependent kinases (CDK). For example, the cyclin-dependent kinase inhibitors, p16, p21, and p27 each regulate cyclin:CDK mediated cell cycle signaling. Overexpression of these factors within a cell by a lentiviral vector particle results in a cytostatic suppression of cell proliferation. Other factors that may be used therapeutically, as blocking agents or targets, include,  
15 for example, wild-type or mutant Rb, p53, Myc, Fos, Jun, PCNA, GAX, and p15.

### 3. Expression of Palliatives

Techniques similar to those described above can be used to produce lentiviral vector particles which direct the expression of an agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present  
20 invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected  
25 cells. Examples of such functions for a cancerous cell, cancer-promoting growth factor, or uncontrolled proliferative condition (*e.g.*, restenosis) include viability, cell replication, altered susceptibility to external signals (*e.g.*, contact inhibition), and lack of production or production of mutated forms of anti-oncogene proteins.

#### a. Inhibitor Palliatives

30 In one aspect of the present invention, the lentiviral vector particle directs the expression of a gene which can interfere with a function of a pathogenic agent, for instance in viral or malignant diseases. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated either with the pathogenic condition or with a specific cell type (an

"identifying agent"). In addition, vector delivery may be controlled by targeting vector entry specifically to the desired cell type (for instance, a virally infected or malignant cell) as discussed above.

One method of administration is leukaphoresis, in which about 20% of an individual's PBLs are removed at any one time and manipulated *in vitro*. Thus, approximately  $2 \times 10^9$  cells may be treated and replaced. Repeat treatments may also be performed. Alternatively, bone marrow may be treated and allowed to amplify the effect as described above. In addition, packaging cell lines producing a vector may be directly injected into a subject, allowing continuous production of recombinant virions.

In one embodiment, lentiviral vector particles which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the lentiviral vector particle would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication.

In a third embodiment, lentiviral vector particles may be introduced which expresses a palliative capable of selectively inhibiting the expression of a pathogenic gene, or a palliative capable of inhibiting the activity of a protein produced by the pathogenic agent. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu<sup>5</sup>" mutant; *see* Wachsmann et al., *Science* 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., *Nature* 335:452, 1988).

Such a transcriptional repressor protein can be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are

introduced to the system, a mutant with the appropriate properties (*i.e.*, represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" lentivirus vector (*i.e.*, one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

b. Conditional Toxic Palliatives

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the lentiviral vector should be limited by the presence of an entity associated with the pathogenic agent, such as a specific viral RNA sequence identifying the pathogenic state, in order to avoid destruction of nonpathogenic cells.

In one embodiment of this method, lentiviral vector particles can be utilized to express a toxic gene (as discussed above) from a cell-specific responsive vector. In this manner, rapidly replicating cells, which contain the RNA sequences capable of activating the cell-specific responsive vectors, are preferentially destroyed by the cytotoxic agent produced by the lentiviral vector particle.

In a similar manner to the preceding embodiment, the lentiviral vector can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the *E. coli* guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (*see* Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987). Conditionally lethal gene products of this type (also referred to as "pro-drugs" or "prodrug activating enzymes") have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a

nontoxic drug which is not a purine or pyrimidine analogue to a cytotoxic form (*see* Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

In another aspect of the present invention, lentiviral vectors are provided which direct the expression of a gene product capable of activating an otherwise  
5 inactive precursor into an active inhibitor of the pathogenic agent. For example, the HSVTK gene product may be used to more effectively metabolize potentially antiviral nucleoside analogues such as AZT or ddC. The HSVTK gene may be expressed under the control of a cell-specific responsive vector and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to  
10 the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus, HIV replication (Furman et al., *Proc. Natl. Acad. Sci. USA* 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleoside and nucleoside kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide triphosphate form. AZT or ddC  
15 therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

20 Administration of these lentiviral vector particles to human T cell and macrophage/monocyte cell lines can increase their resistance to HIV in the presence of AZT and ddC compared to the same cells without retroviral vector treatment. Treatment with AZT would be at lower than normal levels to avoid toxic side effects but still efficiently inhibit the spread of HIV. The course of treatment would be as  
25 described for the blocker.

In one embodiment, the lentiviral vector particle carries a gene specifying a product which is not in itself toxic but, when processed or modified by a protein such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the lentiviral vector could carry a gene encoding a proprotein for  
30 ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxin ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In another embodiment, the lentiviral vector particle may express a  
35 "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as expression of a viral gene). This surface protein



can be recognized by a cytotoxic agent, such as antibodies for the reporting protein, or by cytotoxic T cells. In a similar manner, such a system can be used as a detection system (*see below*) to simply identify those cells having a particular gene which expresses an identifying protein.

5 Similarly, in another embodiment, a surface protein could be expressed which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV *env* intracellularly could inhibit the  
10 formation of viable viral particles, much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV *env* complex has been implicated as a cause  
15 of cell death, additional expression of CD4 (in the presence of excess HIV-*env* present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractivity to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4  
20 on their cell surfaces).

In another embodiment, the lentiviral vector particle can provide a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected cell. By making ribozyme production dependent on a specific RNA sequence corresponding to the pathogenic state, such as HIV *tat*, toxicity is specific to  
25 the pathogenic state.

#### 4. Expression of Markers

The above-described technique of expressing a palliative in a cell in response to a specific RNA sequence can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene  
30 carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), in  
35 a lentiviral vector which responds to the presence of the identifying protein in the

infected cells. For example, HIV, when it infects suitable cells, makes tat and rev. The indicator cells can thus be provided with a genome (such as by infection with an appropriate lentivirus particle) which codes for a marker gene, such as the alkaline phosphatase gene,  $\beta$ -galactosidase gene, or the luciferase gene which is expressed by the lentivirus particle upon activation by the tat and/or rev RNA transcript. In the case of  $\beta$ -galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is positive for HIV. For intracellular enzymes such as  $\beta$ -galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making cell extracts and performing a suitable assay. For the membrane bound form of alkaline phosphatase, virus titre can also be measured by performing enzyme assays on the cell surface using a fluorescent substrate. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of this marker system can be used for different purposes. These include counting active virus, or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and positive where there were no antibodies, this would assist in confirming the presence of HIV.

Within an analogous system for an *in vitro* assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable lentiviral vector particle which carries the reporter gene which is only expressed in the presence of the appropriate viral RNA transcript. The reporter gene, after entering the sample cells, will express its reporting product (such as  $\beta$ -galactosidase or luciferase) only if the host cell expresses the appropriate viral proteins.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of reporting product than identifying agent present, which results in an amplification effect.

### 5. Immune Down-Regulation

As described above, the present invention also provides lentiviral vector particles capable of suppressing one or more elements of the immune system in target cells infected with the lentivirus. Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells, and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with a gp 19-encoding lentiviral vector which, upon expression of the gp 19, inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection. In the context of arthritis, lentiviral vectors may be utilized to directly transduce synoviocytes, either *in vivo*, *ex vivo*.

An alternative method involves the use of anti-sense message, ribozyme, or other specific gene expression inhibitor specific for T cell clones which are autoreactive in nature. These block the expression of the T cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using the viral vector delivery system.

### 6. Replacement or Augmentation Gene Therapy

One further aspect of the present invention relates to transforming cells of a vertebrate or insect with a lentiviral vector which supplies genetic sequences capable of expressing a therapeutic protein. Within one embodiment of the present invention, the lentiviral vector is designed to express a therapeutic protein capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect in metabolism, immune regulation, hormonal regulation, enzymatic or membrane associated structural function. This embodiment also describes the lentiviral vector particle capable of transducing individual cells, whereby the therapeutic protein is able

to be expressed systemically or locally from a specific cell or tissue, whereby the therapeutic protein is capable of (a) the replacement of an absent or defective cellular protein or enzyme, or (b) supplement production of a defective or low expressed cellular protein or enzyme. Such diseases may include cystic fibrosis, Parkinson's disease, hypercholesterolemia, adenosine deaminase deficiency,  $\beta$ -globin disorders, Hemophilia A & B, Gaucher's disease, diabetes and leukemia. Within certain preferred embodiments vectors as described herein are utilized in order to provide long-term expression of the desired gene of interest.

a. Treatment of Gaucher disease

As an example of the present invention, lentiviral vector particles can be constructed and utilized to treat Gaucher disease. Briefly, Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This type of therapy is an example of a single gene replacement therapy by providing a functional cellular enzyme. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuronopathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see *Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677).

b. Lentiviral vector particles Expressing Human Factor VIII and Factor IX for Treatment of Hemophilia

Within one embodiment of the invention, lentiviral vector particles expressing a B-domain deleted factor VIII protein are provided (see also PCT WO 91/09122, and Attorney's Docket No. 1155.005 entitled "Methods for Administration of Recombinant Gene Delivery Vehicles for Treatment of Hemophilia and Other Disorders").

Briefly, the B domain separates the second and third A domains of factor FVIII in the newly synthesized single-chain molecule. The B domain extends from amino acids 712 to 1648 according to Wood *et al.*, 1984, *Nature* 312:330-337. Proteolytic activation of factor FVIII involves cleavage at specific Arg residues located at positions 372, 740, and 1689. Cleavages of plasma factor VIII by thrombin or Factor Xa at Arg 372 and Arg 1689 are essential for factor VIII to participate in coagulation. Therefore, activated factor VIII consists of a heterodimer comprising amino acids

residues 1-372 (containing the A1 domain) and residues 373-740 (containing the A2 domain), and residues 1690-2332 (containing the A3-C1-C2 domain).

An important advantage in using the B domain deleted FVIII molecule is that the reduced size appears to be less prone to proteolytic degradation and therefor, no addition of plasma-derived albumin is necessary for stabilization of the final product. The term "B domain deletion" as used herein with respect to factor VIII protein refers to a factor VIII protein in which some or all removal of some or all of the amino acids between residues 711 and 1694 have been deleted, and which still preserves a biologically active FVIII molecule.

A range of B domain deletions can exist depending on which amino acid residues in the B domain is deleted and whereby the biological activity of the FVIII molecule is still preserved. A specific B domain deletion called the SQN exists which is created by fusing Ser 743 to Gln 1638 (Lind *et al.*, 1995, *Eur J. Biochem* 323:19-27, and PCT WO 91/09122) This deletes amino acid residues 744 to 1637 from the B domain creating a Ser-Glu-Asn (SQN) link between the A2 and A3 FVIII domains. When compared to plasma-derived FVIII, the SQN deletion of the B domain of FVIII did not influence its *in vivo* pharmacokinetics (Fijnvandraat, et al., *P.R.Schattauer Verlagsgesellschaft mbH (Stuttgart)* 77:298-302, 1997). The terms "Factor VIII SQN deletion" or "SQN deletion" as used herein refer to this deletion and to other deletions which preserve the single S-Q-N tripeptide sequence and which result in the deletion of the amino acids between the two B-domain SQN sequences (See PCT WO 91/09122 for a description of this amino acid sequence).

There are number of other B-domain deleted forms of factor VIII. cDNA's encoding all of these B-domain deleted factor VIII proteins can be inserted into lentiviral vector particles by using standard molecular biology techniques. For example cDNA molecules encoding the following B-domain factor VIII deletions can be constructed as described below:

Eaton (1986) Biochemistry 25:8343	des 797-1562 deletion
Toole (1986) PNAS 83:5939	des 760-1639 (LA-FVIII)
Meutien (1988) Prot Eng 2:301	des 771-1666 (FVIII del II: missing one thrombin site)
Sarver (1987) DNA 6:553	des 747-1560
Mertens (1993) Br J Haematol 85:133	des 868-1562 des 713-1637 (thrombin resistant)
Esmon (1990) Blood 76:1593	des 797-1562
Donath (1995) Biochem J 312:49	des 741-1668
Webb (1993) BBRC 190:536	PCR cloned from mRNA
Lind (1995) Eur J Biochem 232:19	des 748-1648 (partially processed) des 753-1648 (partially processed) des 777-1648 (partially processed) des 744-1637 (FVIII-SQ) des 748-1645 (FVIII-RH) des B-domain + 0, 1, 2 Arg (partially processed) desB, +3Arg (FVIIIIR4) desB, +4Arg (FVIIIIR5)
Langner (1988) Behring Inst Mitt 16-25	des 741-1689 des 816-1598
Cheung (1996) Blood 88:325a	des 746-1639
Pipes (1996) Blood 88:441a	des 795-1688 (thrombin sites mutated)

A B domain deletion in which an IgG hinge region has been inserted can also be used. For instance, a deletion of this type can be obtained from plasmid PSVF8-t $\beta$ 2, which was designed to link the heavy and light chains with a short hinge region from immunoglobulin A. To obtain cleavage at the end of the heavy chain and to release the light chain, some residues of the  $\beta$  domain are included on either side of the hinge sequence. The 5' untranslated leader and signal peptide are from the human Factor VIII:C cDNA, with the Kozak consensus sequence at the initiation codon as in pSVF8-302. A description of this vector is included in Chapman *et al.*, U.S. Patent No. 5,595,886. The 3' untranslated region is the same fused Factor VIII and tPA sequence as found in pSVF8-80K.

The construction may be completed in two steps: an oligomer with cohesive ends for EcoRI and BclI (117 bp) was cloned into a transfer vector, pF8GM7, the DNA sequence of the oligomer was checked by m13 subcloning and Sanger sequencing.

Next, the final plasmid was assembled by ligation of the following three fragments:

- (a) FspI-EcoRI fragment from pSVF8-92S;
- (b) EcoRI-NdeI fragment of the transfer vector pF8GM7 with oligomer; and
- (c) FspI-NdeI fragment of pSVF8-80K.

5 Descriptions of pSVF8-92S and pSVF8-80K are included in Chapman *et al.*, U.S. Patent No. 5,595,886.

Three additional B domain-deleted factor VIII constructs of particular interest for inclusion in the lentiviral vector particles of the invention can be prepared as follows. Plasmid pSVF8-500 encodes a factor VIII protein with amino acids 770 to  
10 1656 of the full length Factor VIII deleted. In addition the threonine at position 1672 of the full-length factor VIII sequence was also deleted. The following is a description of the construction of the vector.

The pSVF8-500 plasmid is a derivative of pSVF8-302 in which the regions coding for the 92K and 80K domains are fused with a small connecting  $\beta$ -  
15 region of 21 amino acids, retaining the natural proteolytic processing sites. This plasmid was constructed in the following manner:

(1) A SalI-KpnI fragment of 1984 bp containing the region coding for the 92K protein (except for the carboxyl terminal end) and BstXI-SalI fragment of 2186 bp containing the region coding for the carboxyl end of the 80K protein with 3'  
20 end untranslated region were isolated by gel electrophoresis after digestion of pSVF8-302 with restriction enzymes.

(2) A BclI-BstXI fragment of 1705 bp containing most of the region coding for the 80K protein was isolated after gel electrophoresis of the BamHI-XbaI fragment of pUC12F8. (pUCF812 is prepared from pF8-102 which is described in U.S.  
25 Patent No. 5,045,455. pF8-102 is digested with Bam-XbaI and ligated into vector pUC12 by *in vitro* mutagenesis at a BclI site using the following primer: 5' ACT ACT CTT CAA TCT GAT CAA GAG GAA 3' (Seq ID No. \_\_\_\_).

(3) A KpnI-EcoRI fragment containing the carboxyl end of the 92K protein and part of the  $\beta$  region (4 amino acids) was obtained by digestion of the SalI  
30 cassette from pSVF8-302 with KpnI and EcoRI.

(4) Ligation of four pieces of synthetic DNA (shown in Figure 39) to the fragments of steps (2) and (3) and digestion with KpnI.

(5) Final ligation of fragments from steps (1) and (4); digestion with SalI and gel purification of the 6428 bp SalI cassette.

35 (6) Ligation of the SalI cassette into pSV7d vector; transformation of HB101 and colony hybridization to isolate pSVF8-500 (Figure 40). The sequence of

the junction region coding for 92K- $\beta$ -80K was verified by DNA sequence after cloning in M13.

The sequence was changed to incorporate unique NruI and MluI restriction sites without changing the amino acid sequence. These sites were also used to construct other two additional B-domain deleted vectors which are described below.

pSV500B $\Delta$ Thr was constructed from pSVF8-500. The threonine deletion at position 1672 was maintained. A synthetic linker was used to construct pSV500B $\Delta$ Thr. The linker extends from a unique NruI site at Ser(765) to a unique MluI site at Ile(1659) in the pSVF8-500 vector. This linker was substituted for the corresponding region of pSVF8-500.

A third vector pSVF8-500B was constructed from pSV500B $\Delta$ Thr. This vector is identical to pSVF8-500B except that the codon for threonine 1672 was re-inserted using standard mutagenesis methods. The relationship between, pSVF8-500B, pSVF8-500B, is further illustrated in the table below. Amino acid sequence numbers in the table were determined by reference to full-length factor VIII sequence.

Name	Amino Acids Deleted	Thr at 1672 Deletion
pSVF8-500	770 to 1656	Yes
pSVF8-500B $\Delta$ Thr	779 to 1658	Yes
pSVF80-500B	779 to 1658	No

In all cases, the BglII-PflI 1.35 kb fragments of each modified cDNA listed above can be inserted into the lentiviral vector particles described herein using standard molecular biology procedures known to those of skill in the art and described herein.

The full-length factor VIII cDNA can also be inserted into the lentiviral vector particles of the invention (*see, e.g.*, WO 96/21035). A variety of Factor VIII deletions, mutations, and polypeptide analogs of Factor VIII can also be introduced into the lentiviral vector particles of the invention including lentiviral vector particles by modifications of the procedures described herein. These analogs include, for instance, those described in PCT Patent Publication Nos. WO 97/03193, WO 97/03194, WO 97/03195, and WO 97/03191, all of which are hereby incorporated by reference.

Hemophilia B can also be treated with systemically administered factor IX-expressing lentiviral vector particles including lentiviral vector particles. Human factor IX deficiency (Christmas disease or Hemophilia B) affects primarily males because it is transmitted as sex-linked recessive trait. It affects about 2000 people in the US. The human factor gene codes a 416 amino acids of mature protein.



The human factor IX cDNA can be obtained for instance by constructing plasmid pHIX1, as described by Kurachi and Davie, 1982, *PNAS* 79(21):6461-6464. The cDNA sequence can be excised as a PstI fragment of about 1.5 kb, blunt ended using T4 DNA polymerase. The factor cDNA fragment can be readily inserted, for example into a SrfI site introduced into a lentiviral vector particle.

c. Lentiviral vector particles expressing other clotting factors

i. Factor V.

Lentiviral vector particles can be constructed using molecular biology techniques known to those of skill in the art. For instance, Factor V cDNA is obtained from pMT2-V (Jenny, 1987, *Proc. Natl. Acad. Sci. USA* 84:4846; ATCC deposit #40515) by digestion with Sall. The 7 kb cDNA band is excised from agarose gels and cloned into lentiviral vector particles, using standard molecular biology techniques.

Either a full-length or a B-domain deletion or substitution of the factor V cDNA can be expressed by the gene therapy vectors of the invention. Factor V B-domain deletions such as those reported by Marquette, 1995, *Blood* 86:3026, and Kane, 1990, *Biochemistry* 29:6762, can be made as described by these authors.

ii. Antithrombin III

Lentiviral vector particles capable of expressing ATIII cDNA can be readily constructed using standard molecular biology techniques known to those of skill in the art. For instance a lentiviral vector particle expressing AT III can be constructed from the vector pKT218 (Prochownik, 1983, *J. Biol. Chem.* 258:8389; ATCC number 57224/57225) by excision with PstI. The 1.6 kb cDNA insert can be recovered from agarose gels and cloned into the PstI site of vector SK-. The insert can be recovered by restriction enzyme digestion and cloned into lentiviral vector particles described herein by the restriction enzymes.

iii. Protein C

The lentiviral vector particles of the invention capable of expressing Protein C can be made using a wide variety of techniques given the present disclosure. For instance, protein C cDNA will be obtained by restriction enzyme digestion of published vector (Foster, 1984, *Proc. Natl. Acad. Sci. USA* 81:4766; Beckmann, 1985, *Nucleic Acids Res* 13:5233). The 1.6 kb cDNA insert can be recovered from agarose gels and cloned into the multiple cloning site of vector SK- under standard conditions. The insert can be recovered by restriction enzyme digestion and cloned into a lentiviral

vector particle; for example, excision by XhoI/NotI digestion followed by cloning into XhoI/NotI digested lentiviral vector particle.

iv. Prothrombin

5           Lentiviral vector particles expressing prothrombin and its variants can be constructed by methods known to those of skill in the art, by using variations on the methods described herein. For instance, prothrombin cDNA can be obtained by restriction enzyme digestion of a published vector (Degen (1983) *Biochemistry* 22:2087). The 1.9 kb cDNA insert can be recovered from agarose gels and cloned into  
10   the multiple cloning site of vector SK-. The insert can be recovered by restriction enzyme digestion and cloned into a lentiviral vector particle using restriction enzyme digestion

v. Thrombomodulin

15           Lentiviral vector particles expressing thrombomodulin and its variants can be constructed using techniques known to those of skill in the art. For instance, thrombomodulin cDNA can be obtained from the vector puc19TM15 (Jackman, 1987, *Proc. Natl. Acad. Sci. USA* 84:6425; Shirai, 1988, *J. Biochem.* 103:281; Wen, 1987, *Biochemistry* 26:4350; Suzuki, 1987, *EMBO J* 6:1891; ATCC number 61348,61349) by  
20   excision with SalI. The 3.7 kb cDNA insert can be recovered from agarose gels and cloned into the SalI site of lentiviral vector particle.

d. Lentiviral vector particles treatment of hereditary disorders and other conditions

          There are a number of proteins useful for treatment of hereditary  
25   disorders that can be expressed *in vivo* by the methods of invention. Many genetic diseases caused by inheritance of defective genes result in the failure to produce normal gene products, for example, thalassemia, phenylketonuria, Lesch-Nyhan syndrome, severe combined immunodeficiency (SCID), hemophilia, A and B, cystic fibrosis, Duchenne's Muscular Dystrophy, inherited emphysema and familial  
30   hypercholesterolemia (Mulligan *et al.*, 1993, *Science* 260:926; Anderson *et al.*, 1992, *Science* 256:808; Friedman *et al.*, 1989, *Science* 244:1275). Although genetic diseases may result in the absence of a gene product, endocrine disorders, such as diabetes and hypopituitarism, are caused by the inability of the gene to produce adequate levels of the appropriate hormone insulin and human growth hormone respectively.

Gene therapy by the methods of the invention is a powerful approach for treating these types of disorders. This therapy involves the introduction of normal recombinant genes into somatic cells so that new or missing proteins are produced inside the cells of a patient. A number of genetic diseases can be treated by gene therapy, including adenine deaminase deficiency, cystic fibrosis,  $\alpha_1$ -antitrypsin deficiency, Gaucher's syndrome, as well as non-genetic diseases. Other representative diseases include lactase for treatment of hereditary lactose intolerance, AD for treatment of ADA deficiency, and alpha-1 antitrypsin for treatment of alpha-1 antitrypsin deficiency. See F.D. Ledley, 1987, *J. Pediatrics* 110:157-174; I. Verma, Scientific American (Nov., 1987) pp. 68-84; and PCT Patent Publication WO 95/27512 entitled "Gene Therapy Treatment for a Variety of Diseases and Disorders" for a description of gene therapy treatment of genetic diseases.

One such disorder is familial hypercholesterolemia is a disease characterized clinically by a lifelong elevation of low density lipoprotein (LDL), the major cholesterol-transport lipoprotein in human plasma; Pathologically by the deposition of LDL-derived cholesterol in tendons, skin and arteries leading to premature coronary heart disease; and genetically by autosomal dominant inherited trait. Heterozygotes number about 1 in 500 persons worldwide. Their cells are able to bind cholesterol at about half the rate of normal cells. Their plasma cholesterol levels show two fold elevation starting at birth. Homozygotes number 1 in 1 million persons. They have severe cholesterolemia with death occurring usually before age 20. The disease (Arteriosclerosis) depends on geography. It affects 15.5 per 100,000 individuals in the U.S. (20,000 total) and 3.3 per 100,000 individuals in Japan. Lentiviral vector particles expressing the LDL receptor for treatment of disorders manifesting with elevated serum LDL can be constructed by techniques known to those of skill in the art. An example of a lentiviral vector particle expressing LDL receptor is shown in example 32 herein.

There are a variety of other proteins of therapeutic interest that can be expressed *in vivo* by lentiviral vector particles using the methods of the invention. For instance sustained *in vivo* expression of tissue factor inhibitory protein (TFPI) is useful for treatment of conditions including sepsis and DIC and in preventing reperfusion injury. (See PCT Patent Publications Nos. WO 93/24143, WO 93/25230 and WO 96/06637. Nucleic acid sequences encoding various forms of TFPI can be obtained, for example, as described in US Patent Nos. 4,966,852; 5,106,833; and 5,466,783, and can be incorporated in lentiviral vector particles as is described herein.

Other proteins of therapeutic interest such as erythropoietin (EPO) and leptin can also be expressed *in vivo* by lentiviral vector particles according to the

methods of the invention. For instance EPO is useful in gene therapy treatment of a variety of disorders including anemia (see PCT publication number WO 95/13376 entitled "Gene Therapy for Treatment of Anemia".) Sustained gene therapy delivery of leptin by the methods of the invention is useful in treatment of obesity. (See WO  
5 96/05309 entitled "Obesity Polypeptides able to modulate body weight" for a description of the leptin gene and its use in the treatment of obesity. Lentiviral vector particle expressing EPO or leptin can readily be produced using the methods described herein and the constructs described in these two patent publications.

A variety of other disorders can also be treated by the methods of the  
10 invention. For example, sustained *in vivo* systemic production of apolipoprotein E or apolipoprotein A by the lentiviral vector particles of the invention can be used for treatment of hyperlipidemia. (See Breslow, J. et al. Biotechnology 12, 365 (1994).) In addition, sustained production of angiotensin receptor inhibitor (T.L. Goodfriend, *et al.*, 1996, *N. Engl. J. Med.* 334:1469) can effected by the gene therapy methods described  
15 herein. As yet an additional example, the long term *in vivo* systemic production of angiotatin by the lentiviral vector particles of the invention is useful in the treatment of a variety of tumors. (See O'Reilly *et al.*, 1996, *Nature Med.* 2:689.

#### 7. Lymphokines and Lymphokine Receptors

As noted above, the present invention also provides lentiviral vector  
20 particles which can, among other functions, direct the expression of one or more cytokines or cytokine receptors. Briefly, in addition to their role as cancer therapeutics, cytokines can have negative effects resulting in certain pathological conditions. For example, most resting T-cells, B cells, large granular lymphocytes and monocytes do not express IL-2R (receptor). In contrast to the lack of IL-2R expression on normal  
25 resting cells, IL-2R is expressed by abnormal cells in patients with certain leukemias (ATL, Hairy-cell, Hodgkins, acute and chronic granulocytic), autoimmune diseases, and is associated with allograft rejection. Interestingly, in most of these patients the serum concentration of a soluble form of IL-2R is elevated. Therefore, with certain  
embodiments of the invention therapy may be effected by increasing the serum  
30 concentration of the soluble form of the cytokine receptor. For example, in the case of IL-2R, a lentiviral vector can be engineered to produce both soluble IL-2R and IL-2R, creating a high affinity soluble receptor. In this configuration, serum IL-2 levels would decrease, inhibiting the paracrine loop. This same strategy also may be effective against  
autoimmune diseases. In particular, because some autoimmune diseases (*e.g.*,  
35 Rheumatoid arthritis, SLE) also are associated with abnormal expression of IL-2,

blocking the action of IL-2 by increasing the serum level of receptor may also be utilized in order to treat such autoimmune diseases.

In other cases inhibiting the levels of IL-1 may be beneficial. Briefly, IL-1 consists of two polypeptides, IL-1 and IL-1, each of which has pleiotropic effects. IL-1 is primarily synthesized by mononuclear phagocytes, in response to stimulation by microbial products or inflammation. There is a naturally occurring antagonist of the IL-1R, referred to as the IL-1 Receptor antagonist ("IL-1Ra"). This IL-1R antagonist has the same molecular size as mature IL-1 and is structurally related to it. However, binding of IL-1Ra to the IL-1R does not initiate any receptor signaling. Thus, this molecule has a different mechanism of action than a soluble receptor, which complexes with the cytokine and thus prevents interaction with the receptor. IL-1 does not seem to play an important role in normal homeostasis. In animals, antibodies to IL-1 receptors reduce inflammation and anorexia due to endotoxins and other inflammation inducing agents.

In the case of septic shock, IL-1 induces secondary compounds which are potent vasodilators. In animals, exogenously supplied IL-1 decreases mean arterial pressure and induces leukopenia. Neutralizing antibody to IL-1 reduced endotoxin-induced fever in animals. In a study of patients with septic shock who were treated with a constant infusion of IL-1R for three days, the 28 day mortality was 16% compared to 44% in patients who received placebo infusions. In the case of autoimmune disease, reducing the activity of IL-1 reduces inflammation. Similarly, blocking the activity of IL-1 with recombinant receptors can result in increased allograft survival in animals, again presumably by decreasing inflammation.

These diseases provide further examples where lentiviral vector particles may be engineered to produce a soluble receptor or more specifically the IL-1Ra molecule. For example, in patients undergoing septic shock, a single injection of IL-1Ra producing vector particles could replace the current approach requiring a constant infusion of recombinant IL-1R.

Cytokine responses, or more specifically, incorrect cytokine responses may also be involved in the failure to control or resolve infectious diseases. Perhaps the best studied example is non-healing forms of leishmaniasis in mice and humans which have strong, but counterproductive  $T_H2$ -dominated responses. Similarly, lepromatous leprosy is associated with a dominant, but inappropriate  $T_H2$  response. In these conditions, lentiviral vector particles may be useful for increasing circulating levels of IFN gamma, as opposed to the site-directed approach proposed for solid tumor therapy. IFN gamma is produced by  $T_H1$  T-cells, and functions as a negative regulator

of T<sub>H</sub>-2 subtype proliferation. IFN gamma also antagonizes many of the IL-4 mediated effects on B-cells, including isotype switching to IgE.

IgE, mast cells and eosinophils are involved in mediating allergic reaction. IL-4 acts on differentiating T-cells to stimulate T<sub>H</sub>-2 development, while  
5 inhibiting T<sub>H</sub>-1 responses. Thus, lentivirus-based gene therapy may also be accomplished in conjunction with traditional allergy therapeutics. One possibility is to deliver lentiviral vector particles which produces IL4R with small amounts of the offending allergen (*i.e.*, traditional allergy shots). Soluble IL-4R would prevent the activity of IL-4, and thus prevent the induction of a strong T<sub>H</sub>-2 response.

10 a. Lentiviral vector particles for treatment of viral hepatitis

The lentiviral vector particles including lentiviral vector particles and the methods of administration described are useful for treatment of viral hepatitis, including hepatitis B and hepatitis C. For instance, the lentiviral vector particles of the invention can be used to express interferon-alpha for treatment of viral hepatitis. While not  
15 wishing to be bound by theory, lentiviral vector particles injected intravenously preferentially transduce liver cells. Thus, the methods of intravenous delivery described herein for lentiviral vector particles can be used for treatment of liver diseases such as hepatitis and in particular viral hepatitis, in which therapeutic proteins expressed by the lentiviral vector particles such as lentiviral vector particles can be delivered  
20 preferentially to the liver.

Currently, the only approved treatment for chronic hepatitis B, C and D infections is the use of alpha interferon 2a and 2b. Alpha-interferon is a secreted protein induced in B lymphocytes, macrophages and null lymphocytes by foreign cells, virus-infected cells, tumor cells, bacterial cells and products and viral envelopes. The  
25 mechanism of antiviral action of interferon is by inducing the synthesis of effector proteins: two of the most important are 2', 5'-oligo-adenylate synthetase (OAS) and dsRNA-dependent protein kinase (RDPK). OAS synthesizes adenylylate oligomers that activate RNAaseL, which degrades viral single stranded RNA. RDPK phosphorylates initiation factor eIF-2a which results in the inhibition of viral protein translation. In  
30 addition to the direct antiviral effect, alpha interferon has immunomodulatory effects that are important against viral infections. These immunomodulatory effects are: enhancement of the expression of both Class I and class II major histocompatibility complex (MHC) molecules, modulation of the expression of the interleukin-2 receptor, TNF- $\alpha$  receptor, transferrin receptor, enhancement of spontaneous natural killer (NK)  
35 cell cytotoxicity and modulation of antibody production by B cells. In chronic hepatitis

B infection, the beneficial effect of interferon alpha appears to be from the immunomodulatory effects, while in chronic hepatitis C infection, the beneficial effect is dependent on its antiviral activity. (Bresters, D., in *Hepatitis C Virus*, pp121-136, Reesink HW (ed), 1994). The mechanism of action in interferon alpha for treatment of  
5 chronic hepatitis D is poorly understood (Rizzetto, M. and Rosina, F. in *Viral Hepatitis*, pp. 363-369, Zuckerman, A. J. and Thomas H. C. (ed), 1993).

Localized expression of interferon alpha in the liver from a lentiviral vector particle such as a lentiviral vector particle can be an effective treatment for hepatitis. While not wishing to bound by theory, delivery of alpha interferon at the site  
10 of infection by the gene therapy vectors of the invention, including lentiviral vector particles, results in high local concentration of the cytokine thereby focusing the antiviral and immunological effects to the adjacent infected hepatocytes. A further advantage of this treatment is that the current systemic mode of systemic alpha interferon therapy may either be unnecessary or be reduced in dose and frequency of  
15 treatment. This reduction can reduce the adverse side effects associated with the systemic delivery of alpha interferon. Thus, the gene therapy approaches described herein may be used in combination with administration of alpha-interferon protein formulations.

The construction of a number of different lentiviral vector particles  
20 expressing interferon-alpha can be readily accomplished given the disclosure provided herein. There are at least 24 different human alpha interferon genes or pseudogenes. There are two distinct families (I and II); mature human alpha interferon (I) are 166 amino acids long (one is 165 amino acids) whereas alpha interferon (II) have 172 amino acids. Eighteen genes are in the alpha interferon I family, including at least four  
25 pseudogenes. Six genes are in the alpha interferon II family, including five pseudogenes (Callard, R., and Gearing, A., *Cytokine Facts Book*, Academic Press, 1994 pp. 148-154). In Example 33 herein, we use alpha interferon 2a, 2b, 2c, 54 and 76, all members of the alpha interferon (I) family. Similar techniques can be used for inserting other members of the alpha interferon I family (such as alpha interferon F and N) into  
30 lentiviral vector particles. Thus other biologically active forms of alpha-interferon in addition to 2a, 2b, 2c, 54 and 76 as described herein can also be expressed by the lentiviral vector particles of the invention and used for treatment of viral hepatitis.

Patients with viral hepatitis can be treated a combination gene therapy approach. A lentiviral vector particle expressing a protein drug such as alpha-interferon  
35 can be administered intravenously or directly to the liver by methods described herein. This therapeutic approach can be combined with intramuscular delivery of a lentiviral

vector particle expressing a hepatitis B or hepatitis C antigen for inducing a immune response against the hepatitis virus. Specific hepatitis B and C antigens useful in this type of therapy and the construction of lentiviral vector particles expressing such antigens are described herein and in PCT Patent Publication No. WO 93/15207. In addition, molecularly cloned genomes which encode the hepatitis B virus may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (see Figure 3 of Blum *et al.*, 1989, *TIG* 5(5):154-158) in the Bam HI site of pBR322 (Moriarty *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78:2606-2610). (Note that correctable errors occur in the sequence of ATCC No. 45020.)

#### 8. Suicide Vectors

One further aspect of the present invention relates to the use of lentiviral vector suicide vectors to limit the spread of wild-type lentivirus in the packaging/producer cell lines. For example, within one embodiment the lentiviral vector particles contains a prodrug activating enzyme as discussed above which, upon administration of the prodrug (*e.g.*, gancyclovir) results in the death of cells containing the vector particles.

#### 9. Lentiviral vectors to Prevent the Spread of Metastatic Tumors

One further aspect of the present invention relates to the use of lentiviral vector particles for inhibiting or reducing the invasiveness of malignant neoplasms. Briefly, the extent of malignancy typically relates to vascularization of the tumor. One cause for tumor vascularization is the production of soluble tumor angiogenesis factors (TAF) (Pawletz *et al.*, *Crit. Rev. Oncol. Hematol.* 9:197, 1989) expressed by some tumors. Within one aspect of the present invention, tumor vascularization may be slowed utilizing lentiviral vectors to express antisense or ribozyme RNA molecules specific for TAF. Alternatively, anti-angiogenesis factors (Moses *et al.*, *Science* 248:1408, 1990; Shapiro *et al.*, *PNAS* 84:2238, 1987) may be expressed either alone or in combination with the above-described ribozymes or antisense sequences in order to slow or inhibit tumor vascularization. Alternatively, lentiviral vector particles can also be used to express an antibody specific for the TAF receptors on surrounding tissues.



#### 10. Modulation of Transcription Factor Activity

In yet another embodiment, lentiviral vector particles may be utilized in order to regulate the growth control activity of transcription factors in the infected cell. Briefly, transcription factors directly influence the pattern of gene expression through  
5 sequence-specific *trans*-activation or repression (Karin, *New Biologist* 21:126-131, 1990). Thus, it is not surprising that mutated transcription factors represent a family of oncogenes. Lentiviral vector particles can be used, for example, to return control to tumor cells whose unregulated growth is activated by oncogenic transcription factors, and proteins which promote or inhibit the binding cooperatively in the formation of  
10 homo- and heterodimer *trans*-activating or repressing transcription factor complexes.

One method for reversing cell proliferation would be to inhibit the *trans*-activating potential of the *c-myc*/Max heterodimer transcription factor complex. Briefly, the nuclear oncogene *c-myc* is expressed by proliferating cells and can be activated by several distinct mechanisms, including retroviral insertion, amplification,  
15 and chromosomal translocation. The Max protein is expressed in quiescent cells and, independently of *c-myc*, either alone or in conjunction with an unidentified factor, functions to repress expression of the same genes activated by the *myc*/Max heterodimer (Cole, *Cell* 65:715-716, 1991).

Inhibition of *c-myc* or *c-myc*/Max proliferation of tumor cells may be  
20 accomplished by the overexpression of Max in target cells controlled by lentiviral vectors. The Max protein is only 160 amino acids (corresponding to 480 nucleotide RNA length) and is easily incorporated into a lentiviral vector either independently, or in combination with other genes and/or antisense/ribozyme moieties targeted to factors which release growth control of the cell.

25 Modulation of homo/hetero-complex association is another approach to control transcription factor activated gene expression. For example, transport from the cytoplasm to the nucleus of the *trans*-activating transcription factor NF-B is prevented while in a heterodimer complex with the inhibitor protein IB. Upon induction by a variety of agents, including certain cytokines, IB becomes phosphorylated and NF-B is  
30 released and transported to the nucleus, where it can exert its sequence-specific *trans*-activating function (Baeuerle and Baltimore, *Science* 242:540-546, 1988). The dissociation of the NF-B/IB complex can be prevented by masking with an antibody the phosphorylation site of IB. This approach would effectively inhibit the *trans*-activation activity of the NF-IB transcription factor by preventing its transport to the nucleus.  
35 Expression of the IB phosphorylation site specific antibody or protein in target cells may be accomplished with an lentivirus gene transfer vector. An approach similar to

the one described here could be used to prevent the formation of the *trans*-activating transcription heterodimer factor AP-1 (Turner and Tijan, *Science* 243:1689-1694, 1989), by inhibiting the association between the *jun* and *fos* proteins.

#### FORMULATION

5           Within other aspects of the present invention, methods are provided for preserving an infectious lentiviral vector particle, such that the lentiviral vector particle is capable of infecting mammalian cells upon reconstitution (see U.S. Serial No. 08/153,342). Briefly, lentiviral vector particle which has been purified or concentrated may be preserved or formulated into a pharmaceutical compound or  
10   medicament by first adding a sufficient amount of a formulation buffer to the media containing the lentiviral vector particle, in order to form an aqueous suspension. The formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. As utilized within the context of the present invention, a "buffering compound" or "buffering component"  
15   should be understood to refer to a substance that functions to maintain the aqueous suspension at a desired pH. The aqueous solution may also contain one or more amino acids.

          The lentiviral vector particle can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude lentiviral  
20   vector particle described above may be clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Northborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the lentiviral vector particle in a more desirable buffered  
25   solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified lentiviral vector particle is eluted. A sufficient amount of formulation buffer is added to this eluate to reach a desired final concentration of the constituents and to minimally dilute the lentiviral vector particle, and the aqueous suspension is then stored, preferably at -70°C or immediately dried. As noted above, the formulation  
30   buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

          The crude lentiviral vector particle can also be purified by ion exchange column chromatography (see U.S. Patent Application Serial No. 08/093,436). In  
35   general, the crude lentiviral vector particle is clarified by passing it through a filter, and

the filtrate loaded onto a column containing a highly sulfonated cellulose matrix. The lentiviral vector particle is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation  
5 buffer is then added, as discussed above, to the purified lentiviral vector particle and the aqueous suspension is either dried immediately or stored, preferably at -70°C.

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Specifically, lyophilization involves the steps of cooling the aqueous suspension below the glass transition  
10 temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized lentivirus. Briefly, aliquots of the formulated lentiviral vector particle are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips  
15 et al. (Cryobiology 18:414, 1981) is used to lyophilize the formulated lentiviral vector particle, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized lentivirus. Once lyophilized, the lentiviral vector particle is stable and may be stored at -20°C to 25°C.

Within the evaporative method, water is removed from the aqueous  
20 suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray drying (EP 520,748). Within the spray drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray drying apparatus are available from a number of manufacturers (*e.g.*, Drytec, Ltd., Tonbridge, England; Lab-  
25 Plant, Ltd., Huddersfield, England). Once dehydrated, the lentiviral vector particle is stable and may be stored at -20°C to 25°C. Within the methods described herein, the resulting moisture content of the dried or lyophilized lentivirus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar™ V1B volumetric titrator, Cherry Hill, NJ), or through a gravimetric method.

30 The aqueous solutions used for formulation, as previously described, are composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the lentiviral vector particle upon freezing and lyophilization, or drying through evaporation. Although a  
35 preferred saccharide is lactose, other saccharides may be used, such as sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition,

combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and mannitol (*e.g.*, a concentration of lactose is 3%-4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight. Preferably, the concentration of the high molecular weight structural additive ranges from 0.1% to 10% by weight.

The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or aspartic acid can also be used. A particularly preferred arginine concentration is 0.1% by weight. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred pH of the lentiviral vector particle formulation is 7.4, and a preferred buffer is tromethamine.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated lentiviral vector particle to an appropriate iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride.

Aqueous solutions containing the desired concentration of the components described above may be prepared as concentrated stock solutions.

One method of preserving lentiviral vector particles in a lyophilized state for subsequent reconstitution comprises the steps of (a) combining an infectious lentiviral vector particle with an aqueous solution to form an aqueous suspension, the aqueous suspension including 4% by weight of lactose, 0.1% by weight of human

serum albumin, 0.03% or less by weight of NaCl, 0.1% by weight of arginine, and an amount of tromethamine buffer effective to provide a pH of the aqueous suspension of approximately 7.4, thereby stabilizing the infectious lentiviral vector particle; (b) cooling the suspension to a temperature of from -40°C to -45°C to form a frozen suspension; and (c) removing water from the frozen suspension by sublimation to form a lyophilized composition having less than 2% water by weight of the lyophilized composition, the composition being capable of infecting mammalian cells upon reconstitution. It is preferred that the lentiviral vector particle be replication defective and suitable for administration into humans upon reconstitution.

It will be evident to those skilled in the art given the disclosure provided herein that it may be preferable to utilize certain saccharides within the aqueous solution when the lyophilized lentivirus is intended for storage at room temperature. More specifically, it is preferable to utilize disaccharides, such as lactose or trehalose, particularly for storage at room temperature.

The lyophilized or dehydrated Lentiviruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted lentivirus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted lentivirus. Lyophilized or dehydrated lentiviral vector particle may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

#### ADMINISTRATION

As noted above, recombinant lentiviral particles of the present invention may be administered to a wide variety of locations including, for example, into sites such as the cerebral spinal fluid, bone marrow, joints, arterial endothelial cells, rectum, buccal/sublingual, vagina, the lymph system, to an organ selected from the group consisting of lung, liver, spleen, skin, blood and brain, or to a site selected from the group consisting of tumors and interstitial spaces. Within other related embodiments, the lentiviral vector particle may be administered intrarticularly, intraocularly, intranasally, sublingually, orally, topically, intravesically, intrathecally, topically, intravenously, intraperitoneally, intracranially, intramuscularly, or subcutaneously.

Other representative routes of administration include gastroscopy, ECRP and colonoscopy, which do not require full operating procedures and hospitalization, but may require the presence of medical personnel.

Considerations for administering the compositions of the present invention include the following:

Oral administration is easy and convenient, economical (no sterility required), safe (over dosage can be treated in most cases), and permits controlled release of the active ingredient of the composition (the lentiviral vector particle). Conversely, there may be local irritation such as nausea, vomiting or diarrhea, erratic absorption for poorly soluble drugs, and the lentiviral vector particle will be subject to "first pass effect" by hepatic metabolism and gastric acid and enzymatic degradation. Further, there can be slow onset of action, efficient plasma levels may not be reached, a patient's cooperation is required, and food can affect absorption. Preferred embodiments of the present invention include the oral administration of lentiviral vector particles that express genes encoding erythropoietin, insulin, GM-CSF cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amyloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and pyrimidines. Preferably, the lentiviral vector particles are first lyophilized, then filled into capsules and administered.

Buccal/sublingual administration is a convenient method of administration that provides rapid onset of action of the active component(s) of the composition, and avoids first pass metabolism. Thus, there is no gastric acid or enzymatic degradation, and the absorption of lentiviral vector particles is feasible. There is high bioavailability, and virtually immediate cessation of treatment is possible. Conversely, such administration is limited to relatively low dosages (typically about 10-15 mg), and there can be no simultaneous eating, drinking or swallowing. Preferred embodiments of the present invention include the buccal/sublingual administration of lentiviral vector particles that contain genes encoding self and/or foreign MHC, or

immune modulators, for the treatment of oral cancer; the treatment of Sjogren's syndrome via the buccal/sublingual administration of such lentiviral vector particles that contain IgA or IgE antisense genes; and, the treatment of gingivitis and periodontitis via the buccal/sublingual administration of IgG or cytokine antisense genes.

Rectal administration provides a negligible first pass metabolism effect (there is a good blood/lymph vessel supply, and absorbed materials drain directly into the inferior vena cava), and the method is suitable of children, patients with emesis, and the unconscious. The method avoids gastric acid and enzymatic degradation, and the ionization of a composition will not change because the rectal fluid has no buffer capacity (pH 6.8; charged compositions absorb best). Conversely, there may be slow, poor or erratic absorption, irritation, degradation by bacterial flora, and there is a small absorption surface (about  $0.05\text{m}^2$ ). Further, lipidophilic and water soluble compounds are preferred for absorption by the rectal mucosa, and absorption enhancers (*e.g.*, salts, EDTA, NSAID) may be necessary. Preferred embodiments of the present invention include the rectal administration of lentiviral vector particles that contain genes encoding colon cancer antigens, self and/or foreign MHC, or immune modulators.

Nasal administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. In a preferred embodiment, nasal administration is useful for lentiviral vector particle administration wherein the lentiviral vector particle is capable of expressing a polypeptide with properties as described herein. Conversely, such administration can cause local irritation, and absorption can be dependent upon the state of the nasal mucosa.

Pulmonary administration also avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. Further, pulmonary administration permits localized actions that minimize systemic side effects and the dosage required for effectiveness, and there can be rapid onset of action and self-medication. Conversely, at times only a small portion of the administered composition reaches the bronchioli/alveoli, there can be local irritation, and overdosing is possible. Further, patient cooperation and understanding is preferred, and the propellant for dosing may have toxic effects. Preferred embodiments of the present invention include the pulmonary administration of lentiviral vector particles that express genes encoding IgA or IgE for the treatment of conditions such as asthma, hay fever, allergic alveolitis or fibrosing alveolitis, the CFTR gene for the treatment of cystic fibrosis, and protease and collagenous inhibitors such as  $\alpha$ -1-antitrypsin for the treatment of emphysema.

Alternatively, many of the same types of polypeptides or peptides listed above for oral administration may be used..

Ophthalmic administration provides local action, and permit prolonged action where the administration is via inserts. Further, avoids first pass metabolism, and gastric acid and enzymatic degradation, and permits self-administration via the use of eye-drops or contact lens-like inserts. Conversely, the administration is not always efficient, because the administration induces tearing. Preferred embodiments of the present invention include the ophthalmic administration of lentiviral vector particles that express genes encoding IgA or IgE for the treatment of hay fever conjunctivitis or vernal and atopic conjunctivitis; and ophthalmic administration of lentiviral vector particles that contain genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Transdermal administration permits rapid cessation of treatment and prolonged action leading to good compliance. Further, local treatment is possible, and avoids first pass metabolism, and gastric acid and enzymatic degradation. Conversely, such administration may cause local irritation, is particularly susceptible to tolerance development, and is typically not preferred for highly potent compositions. Preferred embodiments of the present invention include the transdermal administration of lentiviral vector particles that express genes encoding IgA or IgE for the treatment of conditions such as atopic dermatitis and other skin allergies; and transdermal administration of lentiviral vector particles encoding genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Vaginal administration provides local treatment and one preferred route for hormonal administration. Further, such administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is preferred for administration of compositions wherein the lentiviral vector particles express peptides. Preferred embodiments of the present invention include the vaginal administration of lentiviral vector particles that express genes encoding self and/or foreign MHC, or immune modulators. Other preferred embodiments include the vaginal administration of genes encoding the components of sperm such as histone, flagellin, etc., to promote the production of sperm-specific antibodies and thereby prevent pregnancy. This effect may be reversed, and/or pregnancy in some women may be enhanced, by delivering lentiviral vector particles encoding immunoglobulin antisense genes, which genes interfere with the production of sperm-specific antibodies.



Intravesical administration permits local treatment for urogenital problems, avoiding systemic side effects and avoiding first pass metabolism, and gastric acid and enzymatic degradation. Conversely, the method requires urethral catheterization and requires a highly skilled staff. Preferred embodiments of the present invention include intravesical administration of lentiviral vector particle encoding antitumor genes such as a prodrug activation gene such thymidine kinase or various immunomodulatory molecules such as cytokines.

Endoscopic retrograde cystopancreatography (ERCP) (goes through the mouth; does not require piercing of the skin) takes advantage of extended gastroscopy, and permits selective access to the biliary tract and the pancreatic duct. Conversely, the method requires a highly skilled staff, and is unpleasant for the patient.

Many of the routes of administration described herein (*e.g.*, into the CSF, into bone marrow, into joints, intravenous, intra-arterial, intracranial intramuscular, subcutaneous, into various organs, intra-tumor, into the interstitial spaces, intra-peritoneal, intralymphatic, or into a capillary bed) may be accomplished simply by direct administration using a needle, catheter or related device. In particular, within certain embodiments of the invention, one or more dosages may be administered directly in the indicated manner at dosages greater than or equal to  $10^5$ ,  $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$ ,  $10^{10}$  or  $10^{11}$  cfu.

Lentiviral vector particle may be delivered to the target from outside of the body (as an outpatient procedure) or as a surgical procedure, where the vector is administered as part of a procedure with other purposes, or as a procedure designed expressly to administer the vector. Other routes and methods for administration include the non-traumatic routes disclosed within PCT/US95/16967, as well as administration via multiple sites as disclosed within PCT/US95/16471.

The following examples are offered by way of illustration, and not by way of limitation.

## EXAMPLES

### EXAMPLE 1

#### CONSTRUCTION OF AN HIV-1 BASED VECTOR

##### 5 A. Construction of a vector backbone

Plasmid pSK-ΔH3 is first constructed by digestion, filling and blunt end ligation of the unique *Hind*III site of pBluescript SK(-) (Stratagene, La Jolla, CA).

The HIV-1 LTR from the molecular clone pNL4-3 (Adachi et al., *J. Virol.* 1986, 59, 284/ NIH AIDS Research and Reference reagent program catalog #114) is amplified by Polymerase Chain Reaction (PCR) using oligomers LTRB5 : 5' TAG GAT CCT GGA AGG GCT AAT TTG G 3' (Sequence ID No. \_\_\_\_ ) and LTRB3 : 5' TAG GAT CCT TTC GCT TTC AAG TCC C 3' (Sequence ID No. \_\_\_\_ ), which both display a *Bam*HI restriction site at their 5' end. The amplified fragment of 681 bp in length is cloned into the unique *Bam*HI site of pSK-ΔH3. The resulting plasmid is  
15 named pHIV-LTR.

The 1181 bp *Hind*III to *Hind*III fragment of pNL4-3, comprising the 3' end of the 5' LTR and the 5' part of the *gag* gene is cloned into the unique *Hind*III site of pbluescript SK(-) to create pHIV-H3F5'.

The 916 bp *Hind*III to *Sph*I fragment from pHIV-H3F5' is ligated with  
20 the 3482 bp *Hind*III to *Eco*RI fragment from pHIV-LTR, using a *Sph*I-*Eco*RI adaptor to give pHIV-LTR5'.

In order to abolish the expression of the *gag* gene portion present in the pHIV-LTR5' construct, the initiation codon region is modified from GAG ATG GGT to GAG AACCGG T (Sequence ID No. \_\_\_\_ ) by site directed mutagenesis (Muta-gene kit, Biorad, Hercules, CA) using the mutated oligonucleotide 5' GGA GGC TAG AAG  
25 GAG AGA GAA CCG GTG CGA GAG CGT CG 3' (Sequence ID No. \_\_\_\_ ). This modification results in the destruction of the ATG codon, as well as a +1 frameshift in the open reading frame of the HIV *gag* gene. The mutation also allows the creation of a unique *Age* I site (ACCGGT).

30 In order to delete undesirable restriction sites, the region comprised between the *Eco*RI to *Xho* I sites of plasmid pHIV-LTR is destroyed by digestion with the corresponding enzymes, filling and subsequent religation to give pHIV-LTR-ΔRI/*Xho*.

The 719 bp *Xho* I to *Hind*III fragment from pNL4-3 is ligated with the 3067 bp *Xba* I to *Hind*III fragment from pHIV-LTR-ΔRI/*Xho* using a *Xba* I-*Xho* I adaptor to give pLTR-HIV3'.

To create the HIV-1-based vector backbone, named vHIV-I, the 1510 bp Not I to *Xho* I fragment from pHIV-LTR5' is inserted into the Not I and *Xho* I sites of pHIV-LTR3' (see Figure 1).

#### B. Addition of nuclear transport elements

In order to allow the vector transcript to be efficiently translocated from the cell nucleus to the cytoplasm, nuclear transport elements can be added to vHIV.

10 The HIV-1 Rev-responsive element (RRE) is amplified by PCR from pNL4-3 using the oligomers RRE1 : 5' GCA AGC TTC TGC AGA GCA GTG GGA ATA GG 3' (Sequence ID No. \_\_\_) and RRE2 : 5' GCA AGC TTA CCC CAA ATC CCC AGG AGC TG 3' (Sequence ID No. \_\_\_), which harbor the *Hind* III site at their 5' end. The resulting amplified fragment of 283 bp in length is inserted into the Bam HI  
15 site of pSK(-) to create pRRE.

The RRE fragment is extracted from pRRE using the enzymes *Eco* RI and *Cla* I and inserted into the *Eco* RI and *Cla* I sites of vHIV to give vHIVR.

The Hepatitis B Virus (HBV) posttranscriptional regulatory element (PRE) (Liang and Huang, *Mol. Cell. Biol.* 13:7476, 1993) or the Mason-Pfizer monkey  
20 Virus (MPMV) constitutive transport element (CTE) (Bray et al., *Proc. Natl. Acad. Sci. USA* 91:1256, 1994) are amplified by PCR using specific primers which also display *Hind* III restriction sites at their 5' extremities and inserted in place of the HIV-1 RRE in the vHIVR into the *Eco* RI and *Cla* I sites to give vHIVP and vHIVC respectively.

#### C. Addition of the gene of interest sequence to the vector backbone.

25 A 2kb *Xba*I-*Sst*I fragment from pSP6-βgal (Shapira et al., *Gene* 25:71-82, 1983) is ligated together with a 1.1 kb *Sst* I - *Sma* I fragment from pSP6-βgal and a 2.6 kb *Xba* I - *Sma* I digested pUC19 DNA to form pUC-βgal. The beta-galactosidase gene is then extracted from pUC-beta-Gal by cutting with the restriction enzymes *Sal* I and *Sma* I. A 750 bp fragment containing the human Cytomegalovirus (hCMV) early  
30 genes promoter is also extracted from pCMV-G (Yee et al., *Proc. Natl. Acad. Sci. USA*, 91:9564, 1994) by cutting with *Xba* I and *Sal* I. These two fragments are ligated together and inserted into the *Xba* I and *Sma* I site of pBluescript (SK-) to give pCMV-beta-Gal.

A 3800 bp *Not* I to *Hind* III fragment is extracted from pCMV-beta-Gal and inserted into the *Xho* I site of vHIVR, after the sites are blunt-ended, to give vHRC-beta-Gal. The ligation of the blunt-ended sites *Not* I and *Xho* I allow to recreate a *Xho* I site. The same cloning strategy is applied to vHIVC to create vHCC-beta-Gal, and to  
5 vHIVP to create vHPC-beta-gal).

#### D. Tissue-specific promoters

To restrict expression of the gene of interest in certain cell-types, the beta-galactosidase gene is put under the control of two different tissue-specific promoters.

10 More specifically, in order to induce liver tissue specific expression of the transgene, the 88 bp *Hinc* II to *Rsa* I fragment from the construct pΔCCAT7, (Yee, Science, 1989, 246, 658), which contains the Hepatitis B Virus (HBV) liver specific enhancer, is cloned upstream the Herpes simplex virus (HSV) thymidine kinase (TK) gene promoter to drive the expression of the beta-galactosidase gene in place of the  
15 CMV promoter.

Similarly, the CD2 gene enhancer (Lake et al., *EMBO J.* 9:3129, 1990) is cloned upstream the HSV TK gene promoter to induce lymphocyte-specific expression.

#### E. Expression of a dicistronic cassette

In order to achieve the expression of two or more transgenes driven by  
20 the same promoter from a single vector, two types of dicistronic constructs are designed.

In the first construct the 2.1 kb *Bam* HI to *Hind* III fragment encoding the firefly luciferase gene sequence (luc) and the Neomycin phosphotransferase gene sequence (neo) distant of 78 nucleotides from pLL78NL (Levine et al., *Gene* 108:167,  
25 1991), is inserted in the *Sal* I to *Sal* I backbone of VHRC-beta-gal to create vHRCL78N. The 78 nucleotide linker between the two genes allows reinitiation of translation of the second open reading frame before the large ribosome subunit detaches from the mRNA.

In the second type of construct, the poliovirus internal ribosome entry  
30 site (PO-IRES) is inserted in between the two gene sequences (Adam, *J. Virol.* 65, 4985, 1991). The IRES allows initiation of the second open reading frame independently of that of the first one.

EXAMPLE 2  
PACKAGING CELL LINE GENERATION

A. Construction of a gag/pol expression cassette: pCMV-HIV-1

To construct the packaging cassette pCMV-HIV-1, the 0.7-kilobase pair  
5 (kb) *Bam*HI-*Sph*I fragment with a 19-base pair (bp) deletion in the putative packaging  
signal of pCMVDP1DenvpA ( Parolin et al., *J. Virol.* 68:3888-3895, 1994) was fused  
with the 8-kb *Sph*I-*Hind*III (from position 1447 to 9606) fragment of pNL4-3  
(containing a full-length infectious HIV-1 genome, Adachi et al., *J. Virol.* 59:284, 1986,  
obtained from NIH AIDS Reagent Program) and the 4-kb *Sall*-*Eco*RI fragment from  
10 pCMV-G (Yee et al., *Proc. Natl. Acad. Sci. USA* 91:9564-9568, 1994). In addition, a  
deletion of the 580-bp *Bgl*II (at position 7031 in pNL4-3)-*Bgl*II (at position 7611 in  
pNL4-3) fragment was created in the HIV-1 Env coding region to eliminate the  
expression of this protein and reduce the potential of generating helper virus during  
vector production.

15 1. Generation of nef(-) mutants

To generate pCMV-HIVnef(-), the sequences between *Hpa*I (at position  
8650 in pNL4-3) and *Hind*III (at position 9606 in pNL4-3) of pCMV-HIV-1 was  
deleted.

20 2. Generation of vif(-) mutants

To generate pCMV-HIVvif(-), pCMV-HIV-1 was digested with *Nde*I (at  
position 5123 in pNL4-3) and repaired with the Klenow fragment to create a 2-bp  
insertion in the coding region of the *vif* gene.

3. Generation of vpu(-) mutants

To generate pCMV-HIVvpu(-), the initiation codon of Vpu was mutated  
25 by site directed mutagenesis (Mutagene kit, Biorad, Hercules, CA) using the  
oligonucleotide 5'TGCTACTATTATAGGTTGTACATGTACTACTTACTG3'.

#### 4. Generation of vpr(-) mutants

To generate pCMV-HIVvpr(-), the pCMV-HIV-1 was digested with *EcoRI* (at position 5747 in pNL4-3) and repaired with the Klenow fragment to generate a 4-bp insertion in the coding region of the *vpr* gene.

#### 5. Generation of vpr(-) nef(-) double mutant

The pCMV-HIVvpr(-)nef(-) double mutant was generated by digesting the pCMV-HIVnef(-) with *EcoRI* and repaired with the Klenow fragment as described above.

#### 4. Generation of pCH-GP-1

To generate pCH-GP-1, the 0.66-kb fragment between position 766 and the *SphI* site at position 1447 in pNL4-3 was amplified by polymerase chain reaction (PCR) using the oligonucleotides Gag5' 5'GAGGATCCTAGAAGGA-GAGAGATGGGT3' and Gag3' 5'GAGGATCCAATAGGCCCTGCATGCACTG3'. The resulting fragment was ligated with the 3.7-kb *SphI-NdeI* fragment from pNL4-3 and the 4-kb *Sall-EcoRI* fragment from pCMV-G.

### B. Addition of nuclear transport signals

#### 1. Addition of RRE

To insert the RRE sequence into pCH-GP-1 for efficient transportation of the gag/pol transcripts from nucleus to cytoplasm, the RRE (between positions 7754 and 8013 in HXB-2 (33)) was amplified by PCR from pv653RSN (31) using the oligonucleotides RRE5 5'GCAAGCTTCTGCAGAGCA-GTGGGAATAGG3' and RRE3 5' GCAAGCTTAC CCCAAATCCC CAGGAGCTG 3' and cloned immediately after the *gag/pol* gene in pCHGP-1. This construct was designated pCH-GP-2 (Figure 3A).

#### 2. Addition of PRE

To insert the HBV PRE sequence for efficient transportation of the gag/pol transcripts, the 650-bp *StuI-HindIII* HBV fragment spanning the PRE region (Liang & Huang, *Mol. Cell. Biol.* 13:7476, 1993; Huang & Yen, *J. Virol.* 68:3193, 1994; Donello et al., *J. Virol.* 70:4345, 1996) in pCCAT-1 (Yee, *Science* 246:658, 1989) is inserted into the *EcoRV* site of pBluescript SK(-) to form pSK-PRE. The PRE

fragment in pSK-PRE is isolated by ClaI and EcoRI digestion and cloned into the ClaI and EcoRI-digested pCH-GP-1 to form pCH-GP-3 (Figure 3B).

### 3. Addition of CTE

To insert the CTE sequence from Mason-Pfizer monkey virus (MPMV) (Bray et al., *Proc. Natl. Acad. Sci. USA* 91:1256, 1994) into pCH-GP-1, the CTE sequence in pGem7fz(-)MPMV is amplified by PCR using a pair of primers, 5' GTCATCGATA GACTGGACAG CCAATG 3' (Sequence ID No. \_\_\_\_ ) and 5' CTAGAATTCC CAAGACATCA TCCGGG3' (Sequence ID No. \_\_\_\_ ), containing the ClaI and the EcoRI, respectively, as described above. The PCR product is inserted into the ClaI and EcoRI-digested pCH-GP-1 to form pCH-GP-4 (Figure 3C).

### C. Generation of stable packaging cell lines

To establish stable packaging cell lines, either the amphotropic env or the VSV-G can be expressed instead of the HIV env to pseudotype the HIV vectors. The first strategy is to establish 293 cell lines expressing the HIV tat necessary for efficient expression of the vector genomic RNA. To construct the tat expression plasmid, a 360-bp SalI-BamHI fragment spanning the first exon of the tat gene is isolated from pCV1 (Arya et al., *Science* 229:69, 1985). A 1390-bp XbaI-XhoI fragment containing the CMV promoter and the rabbit  $\beta$ -globin gene splice signal is isolated from pCMV-G. A 3295-bp BamHI-XbaI fragment containing the polyadenylation site of the rabbit  $\beta$ -globin gene is also isolated from pCMV-G. All three fragments are ligated together to form pCMV-tat (Figure 4A). To establish stable tat-expressing cell lines, 293 cells are co-transfected with pCMV-tat and pSV2-gpt (Mulligan & Berg, *Proc. Natl. Acad. Sci. USA* 78:2072, 1981, obtained from ATCC) at a ratio of 10 to 1. The transfected cells are subjected to selection in mycophenolic acid, xanthine and HAT-containing medium. The surviving cells are pooled and clones are isolated by limiting dilution. To screen for tat expression, the culture supernatant from each clone is spotted on a nylon membrane and tat is detected by the ECL Western blotting system (Amersham, Arlington Heights, IL) using an anti-tat antibody (Advanced Biotechnologies Inc., Columbia, MD).

To express the HIV gag/pol, pCH-GP-3 or pCH-GP-4 is co-transfected with pFR400 (*Proc. Natl. Acad. Sci. USA* 80:2495, 1983) into 293/tat cells. The transfected cells are selected in methotrexate (Mtx)-containing medium and cloned by limiting dilution. Mtx-resistant clones are picked and gag/pol expression is assayed by

HIV p24 in culture supernatant. The 293/tat/gag/pol clone expressing the highest level of p24 is expanded and used for stable expression of amphotropic env.

To express the amphotropic env gene, pCMVenv<sup>am</sup>DraI (PCT #WO 92/05266; US patent #5,591,624) is co-transfected with pTK-puro (Chen et al., *Proc. Natl. Acad. Sci. USA* 93:10057, 1996) into the 293/tat/gag/pol cells. The 293/tat/gag/pol/env clones are isolated from puromycin-resistant colonies by limiting dilution. Expression of the env gene is detected by Western blot analysis using, RLV gP69/71, the anti-env polyclonal antibody (Quality Biotech Inc., Camden, NJ). The clone expressing the highest level of env is identified and expanded.

Packaging cell lines expressing VSV-G instead of the amphotropic env are also established. The VSV-G gene is under the control of the inducible promoter of the tet system (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547, 1992, obtained from Bujard) in pTetO-G-2 (Chen et al., *Proc. Natl. Acad. Sci. USA* 93:10057, 1996) since stable VSV-G expression resulted in cell death. To express VSV-G, stable 293 clones expressing tTAER (Chen et al., *Proc. Natl. Acad. Sci. USA* 93:10057, 1996), the transactivator for the inducible promoter, need to be established first. The 293/tat cells described above are transfected with phyg-CMV-tTAER (Chen et al., *Proc. Natl. Acad. Sci. USA* 93:10057, 1996) and hygromycin-resistant clones are isolated by limiting dilution. To identify the clone expressing the highest levels of tTAER, cells derived from these clones are transfected with pUHC13-3 (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547, 1992, obtained from Bujard) in the presence of tetracycline or  $\beta$ -estradiol and the luciferase (lux) activity is determined 48 hours after transfection. The 293/tat/tTAER clone expressing the highest level of lux activity in the presence of  $\beta$ -estradiol is identified and expanded.

To express the HIV gag/pol, pCH-GP-3 or pCH-GP-4 is co-transfected with pFR400 into 293/tat/tTAER cells, and the 293/tat/tTAER/gag/pol clone expressing the highest level of p24 is isolated as described above. To introduce the VSV-G gene, the 293/tat/tTAER/gag/pol cells are transfected with pTetO-G-2 and selected in puromycin-containing medium. The 293/tat/tTAER/gag/pol/G clone expressing low level of VSV-G is isolated by limiting dilution of the puromycin-resistant cells, followed by  $\beta$ -estradiol induction of VSV-G expression and FACS analysis using I1, the monoclonal antibody specific for G (Burns et al., *Proc. Natl. Acad. Sci. USA* 90:8033, 1993). Clones expressing low-level of VSV-G are isolated because high-level VSV-G expression leads to rapid cell death (within 4 to 5 days) whereas low-level VSV-G expression has little effect on cell growth, resulting in an extended duration of



virus harvest ( up to two weeks) (Chen et al., *Proc. Natl. Acad. Sci. USA* 93:10057, 1996).

The packaging cell lines described above express HIV gag and pol using the CTE and PRE sequence which bypass the requirement of rev and RRE for efficient transportation of the gag/pol mRNA from nucleus into cytoplasm. To determine whether rev and RRE can generate higher virus titers, 293/tat/tTAER cells are co-transfected with pCH-GP-2 and pFR400, and the transfected cells are cloned selection in Mtx-containing medium and limiting dilution. Mtx-resistant clones are picked and gag/pol expression is assayed by introducing the rev gene into these clones. To introduce rev, these clones are infected with the KT-1 virus (Chiron Technologies, San Diego, CA) containing the HIV rev gene in a MLV-based vector. HIV p24 in culture supernatant is assayed 48 hours after infection. The 293/tat/tTAER/ gag/pol clone expressing the highest level of p24 upon rev introduction will be expanded and used for stable expression of rev.

Since high-level expression of rev can be toxic to cells, the rev gene is placed under the control of the inducible promoter of the tet system. The 722-bp Bsu36I fragment containing the rev cDNA is isolated from pCV1 and inserted into the unique BamHI site of pUHG10-3 (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547, 1992, obtained from Bujard) to form pTetO-rev (Figure 4B). To introduce the rev gene, pTetO-rev is co-transfected with pTK-phleomycin (Yee, personal communication) into 293/tat/tTAER/gag/pol cells. The clone expressing the highest level of rev is identified by limiting dilution of phleomycin-resistant cells, followed by  $\beta$ -estradiol induction and p24 detection in the culture supernatant.

To express VSV-G or amphotropic env in 293/tat/tTAER/gag/pol/rev cells, the VSV-G gene in pTetO-G-2 or the amphotropic env gene in pCMVenv<sup>am</sup> is introduced into the 293/tat/tTAER/gag/pol/rev cells and clones expressing either VSV-G or env are isolated as described above.

#### D. Accessory Proteins

Genes encoding the accessory protein can be PCR amplified from pNL4-3 and cloned into pCMV-Bam for expression. The plasmid is co-transfected with pCMUIV-K<sup>b</sup> (Song et al., *J. Biol. Chem.* 269:7024, 1994, obtained from Per Peterson, The Scripps Research Institute, La Jolla, CA) containing the murine H-2K<sup>b</sup> cDNA into one of the packaging cell lines described above. Transfected cells are isolated by FACS sorting using Y3, the monoclonal antibody for H-2K<sup>b</sup>. The clone expressing the highest level of the accessory protein is identified using Western blot analysis with the

specific polyclonal antibody (obtained from NIH AIDS Reagent Program). In the case of Vpr expression which is known to block cell cycle at G2 phase (Planelles et al., *J. Virol* 70:2516, 1996), the PCR amplified Vpr cDNA is inserted into pUHG10-3 under the control of the tet inducible promoter as described for the rev expression. The construct is co-transfected with pCMUIV- K<sup>b</sup> into the packaging cell lines and the Vpr-expressing clone is isolated as described above.

Besides 293-based packaging cell lines, human HT1080 and HeLa cells, canine cf2 and D17-based packaging cell lines are established to test viral titer potential with the same strategy described above.

To generate stable VCL, the HIV vector described above is co-transfected with pCMV-G and pCMVΔR9 (Naldini et al., *Science* 272:263, obtained from Verma, The Salk Institute, La Jolla, CA) into 293 cells. Virus harvested 48 hours after transfection is used to infect one of the packaging cell lines described here and VCL clones are isolated by the neo selection or FACS sorting with the antibody specific for the marker gene carried in the vector (β-gal, alkaline phosphatase or nerve growth factor receptor). Titer potential for the clones is determined by infecting HT1080 cells with the harvested virus from each clone, followed by the neo selection or FACS analysis.

### EXAMPLE 3

#### TRANSIENT PRODUCTION OF INFECTIOUS VECTOR PARTICLES

##### A. Protocols

To generate infectious HIV vectors, 293T cells were seeded at a density of  $4 \times 10^6$  cells per 10 cm-diameter culture dish. Infectious vector with all the accessory proteins was generated by cotransfecting 10μg pCMV-HIV-1, 10μg pCMV-G and 20μg pv653CMVb-gal using the calcium phosphate co-precipitation method (Graham and van der Eb, *Virology* 52:456-467, 1973). Culture medium was replaced 6 to 8 hours later and the culture supernatant was collected 18 hours after transfection, filtered through 0.45 μm filters and stored at -80°C. To generate the vector without any accessory protein, 293T cells were cotransfected with the following five plasmids: 8μg pCHGP plasmid series (the gag/pol plasmid), 8μg pCMV-G (VSV-G plasmid), 16μg pv653CMV β-gal (HIV-vector), 4μg pCMV-Tat and 4μg pCMV-Rev. Within certain experiments, 0 to 10 mM Sodium Butyrate is added to the media during transfection.

Utilizing this five-plasmid transfection protocol, all accessory proteins have been eliminated. Moreover, the possibility of generating replication competent virus is greatly reduced.

To determine the vector titer,  $5 \times 10^4$  HT1080 cells were plated in a 12-well plate in the presence of 8  $\mu\text{g/ml}$  polybrene 24 hours prior to infection. The cells were infected overnight with various dilutions of the vector and assayed for the b-gal activity 48 hours after infection.

To assay for  $\beta$ -gal activity, cells were washed once with PBS, fixed in 1.25% glutaraldehyde for 15 min. and stained for 4 hours at  $37^\circ\text{C}$  in a solution containing 5 mM potassium ferri ferrocyanoide, 400  $\mu\text{g/ml}$  X-Gal (GBT, ST Louis, MO) and 1 mM  $\text{MgCl}_2$ .

#### B. Results

Results are shown in Figure 7. Briefly, as can be seen by this table, deletion of the accessory protein from the vector particle appears to have little effect on vector titer.

Results are also shown in Figure 8. Briefly, CTE from Mason Pfizer Monkey Virus can substitute the function of RRE. But RRE and rev together still appear to generate the highest level of gag production.

Results of the five plasmid transfection protocol are shown in Figure 9. Briefly, as noted above, utilizing the five-plasmid transfection protocol all accessory proteins can be eliminated. Moreover, the possibility of generating replication competent virus is greatly reduced.

Results of the use of sodium butyrate in the five plasmid transfection protocol is shown in Figure 9. Briefly, addition of sodium butyrate in the transfection media can enhance vector particle production by greater than 10 fold.

#### EXAMPLE 4

##### EFFECT OF ACCESSORY PROTEINS ON VECTOR INFECTIVITY

To study the ability of the HIV vector to infect quiescent cells and the effect of the accessory proteins on infectivity, HeLa cells were exposed to gamma-irradiation to arrest cells at the G2 phase of the cell cycle. More specifically, proliferating or growth-arrested HeLa cells were transduced with either MLV- $\beta$ -gal, a

$\beta$ -gal gene-containing MLV vector or the HIV-1-based vector v653CMV $\beta$ -gal(+) containing all four accessory proteins or v653CMV $\beta$ -gal(-) containing no accessory protein. Positive cells were scored by X-Gal staining two days after transduction.

Results in HeLa were expressed as the percentage of titers observed with the same virus preparations in growing HT1080. As shown in Figure 10, no significant difference in titer was observed in proliferating or quiescent HeLa cells transduced with either the v653CMV $\beta$ -gal(+) or the v653CMV $\beta$ -gal(-) vector. In contrast, the transduction efficiency of the MLV vector in quiescent cells was reduced more than 2000 fold. Similar results were obtained with irradiated HT1080 cells, transduced with the three vectors (data not shown).

To ascertain the observed  $\beta$ -gal activity is not due to pseudotransduction of the  $\beta$ -gal activity present in the vector preparation, proliferating HeLa cells transduced with the vector were treated with increasing concentrations of 3'-azido-3'-deoxythymidine (AZT). Both the blue cell number and the  $\beta$ -activity in cell extracts decreased with increasing concentrations of AZT (data not shown). These results demonstrate that, in contrast to the MLV vector, HIV-1-based vectors can transduce quiescent cells efficiently and the HIV-1 encoded accessory proteins are not required to transduce these cells.

To test the infectivity of HIV-1-based vectors in other cell types, primary human skin fibroblasts were allowed either to proliferate or to grow to confluency and then infected with the three retroviral vectors described above. Fibroblasts grown to confluency become contact inhibited and arrested in the G0/G1 phase of the cell cycle (data not shown). As shown in Figure 11, the three vectors exhibit similar transduction efficiency in dividing fibroblasts. However, in quiescent cells, MLV- $\beta$ -gal vector transduction dropped to barely detectable levels. The capacity of v653CMV $\beta$ -gal(+) remained unchanged. In contrast, v653CMV $\beta$ -gal(-), defective for the HIV-1 accessory proteins showed a 4- to 7 fold decreased level of efficiency to transduce the contact-inhibited fibroblasts relative to the v653CMV $\beta$ -gal(+) vector. These results suggest that the requirement for accessory proteins for efficient transduction by HIV-1-based vectors is cell-type dependent.

## CLAIMS

1. A lentiviral vector, comprising a 5' lentiviral LTR, a tRNA binding site, a packaging signal, a promoter operably linked to one or more genes of interest, an origin of second strand DNA synthesis and a 3' lentiviral LTR, wherein said lentiviral vector contains a nuclear transport element that is not RRE.
2. The vector according to claim 1 wherein said nuclear transport element is obtained from MPMV or HBV.
3. The vector according to claim 1 wherein said packaging signal is an extended packaging signal.
4. The vector according to claim 1 wherein said promoter is a tissue-specific promoter.
5. The vector according to claim 1 wherein said promoter is a CMV promoter.
6. The vector according to claim 1 wherein said gene of interest is selected from the group consisting of cytokines, factor VIII, factor IX, LDL receptor, prodrug activating enzymes, trans-dominant negative viral or cancer-associated proteins and tyrosine hydroxylase.
7. The vector according to claim 1, further comprising an internal ribosome entry site.
8. The vector according to claim 1 wherein said promoter is operably linked to two genes of interest which are separated by less than 80 nucleotides.
9. The vector according to claim 1 wherein said lentivirus is selected from the group consisting of HIV, HIV-1, HIV-2, FIV and SIV.

10. A gag/pol expression cassette, comprising a promoter and a sequence encoding gag/pol and at least one of vpr, vpu, nef or vif, wherein said promoter is operably linked to gag/pol and vpr, vpu, nef or vif.

11. A tat expression cassette, comprising a promoter and a sequence encoding tat and at least one of vpr, vpu, nef or vif, wherein said promoter is operably linked to tat and vpr, vpu, nef or vif.

12. A rev expression cassette, comprising a promoter and a sequence encoding rev and at least one of vpr, vpu, nef or vif, wherein said promoter is operably linked to rev and vpr, vpu, nef or vif.

13. A VSV-G expression cassette, comprising a promoter and a sequence encoding VSV-G and at least one of vpr, vpu, nef or vif, wherein said promoter is operably linked to VSV-G and vpr, vpu, nef or vif.

14. A host cell containing an expression cassette according to anyone of claims 10 to 13.

15. A packaging cell line, comprising an expression cassette comprising a promoter, a sequence encoding gag/pol, and a nuclear transport element, wherein said promoter is operably linked to said sequence encoding gag/pol.

16. A packaging cell line, comprising an expression cassette comprising a promoter and a sequence encoding tat, wherein said promoter is operably linked to said sequence encoding tat.

17. A packaging cell line, comprising an expression cassette comprising a promoter and a sequence encoding an envelope, wherein said promoter is operably linked to said sequence encoding the envelope.

18. A packaging cell line, comprising an expression cassette comprising a promoter and a sequence encoding rev, wherein said promoter is operably linked to said sequence encoding the rev.

19. The packaging cell line according to any one of claims 15, 16, 17, or, 18, wherein said packaging cell line further comprises a sequence encoding any one or more of *nef*, *vif*, *vpu* or *vpr*.

20. The packaging cell line according to any one of claims 15, 16, 17, or, 18, wherein said expression cassette is stably integrated.

21. The packaging cell line according to any one of claims 15, 16, 17, or, 18, wherein said cell line, upon introduction of a lentiviral vector, produces particles at a concentration of greater than  $10^5$  cfu/ml.

22. The packaging cell line according to any one of claims 15, 16, 17, or, 18, wherein said promoter is inducible.

23. The packaging cell line according to claim 17 wherein said envelope is VSV-G.

24. The packaging cell line according to any one of claims 15, 16, 17, or, 18, wherein said cell line, upon introduction of a lentiviral vector, produces particles that are free of replication competent virus.

25. A packaging cell line comprising an expression cassette which directs the expression of a *gag/pol* gene, an expression cassette which directs the expression of an *env* gene, an expression cassette which directs the expression of Tat, and expression cassette which directs the expression of Rev.

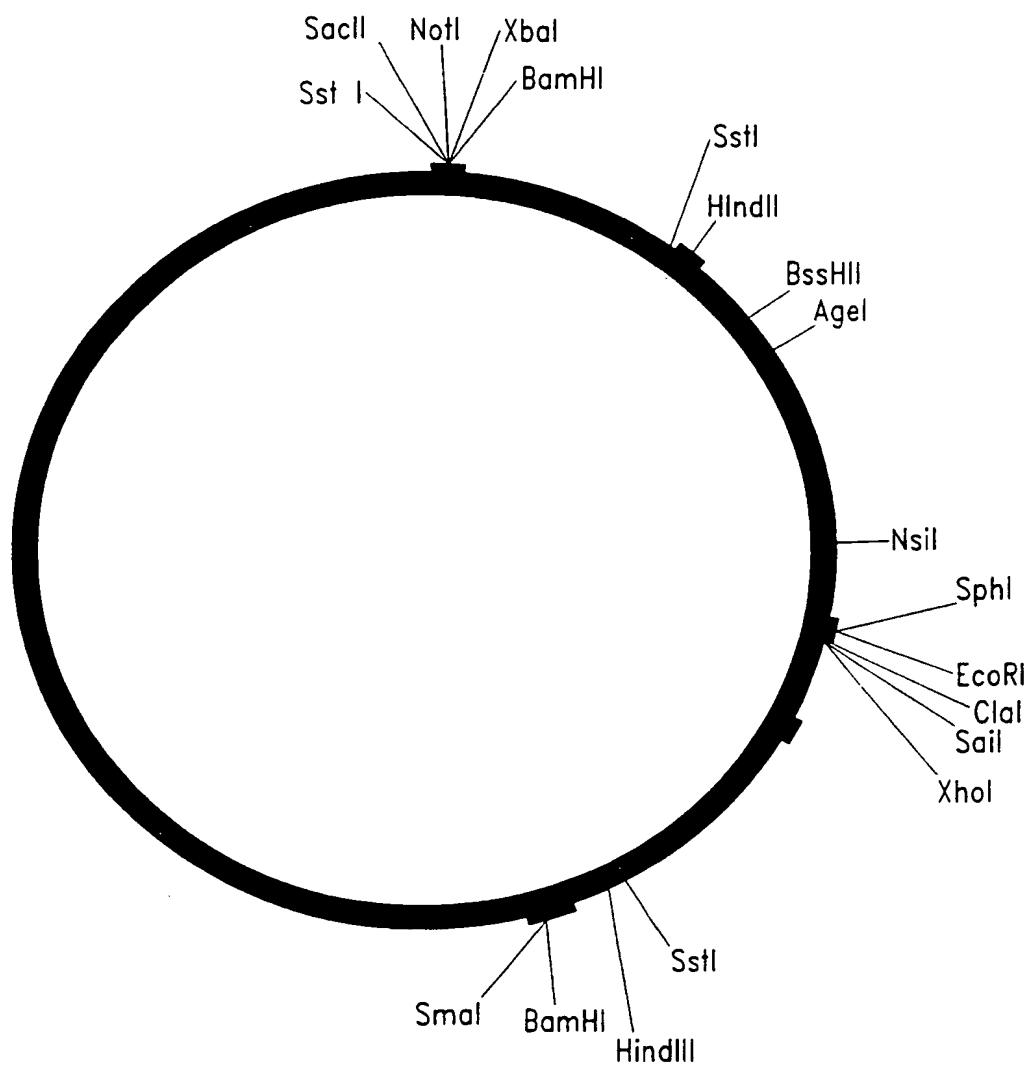
26. The packaging cell line according to claim 25, wherein said *env* gene encodes VSV-G.

27. A vector producing cell line, comprising a packaging cell line according to claim 25 and a lentiviral vector.

28. A method for enhancing production of infectious virus, comprising the step of infecting packaging cell lines with a viral vector, wherein a butyrate salt is added subsequent to, or after infection of the packaging cell line

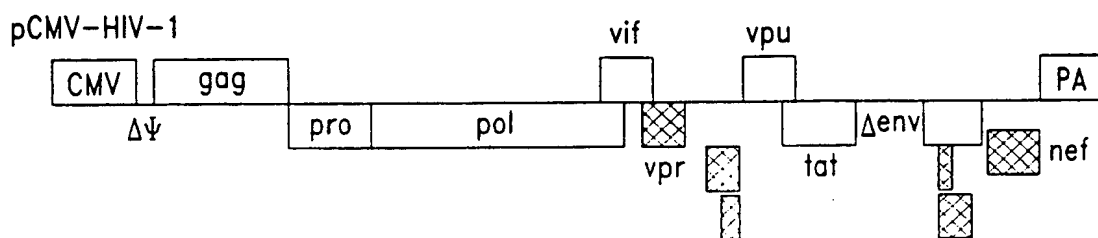
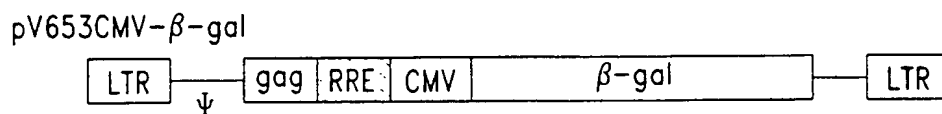
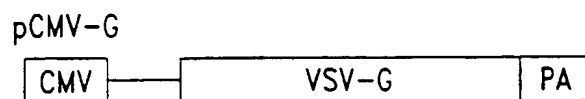
29. The method according to claim 28 wherein said butyrate salt is sodium butyrate.





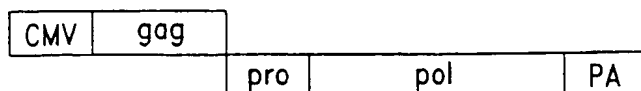
*Fig. 1*

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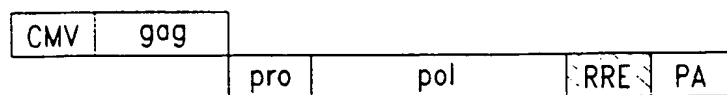
*Fig. 2A**Fig. 2B**Fig. 2C*

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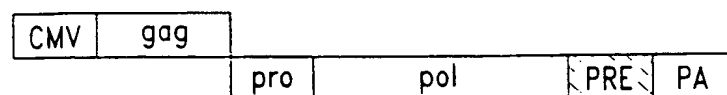
pCHGP-1

*Fig. 3A*

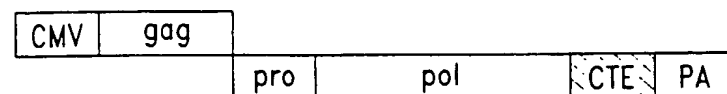
pCHGP-2

*Fig. 3B*

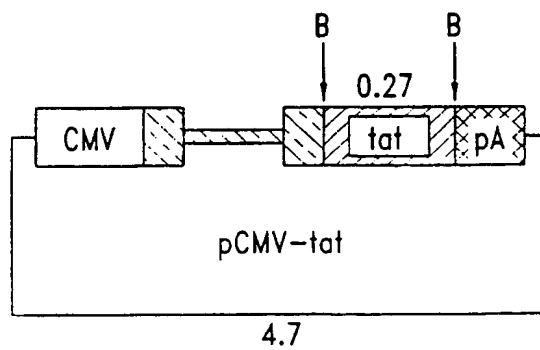
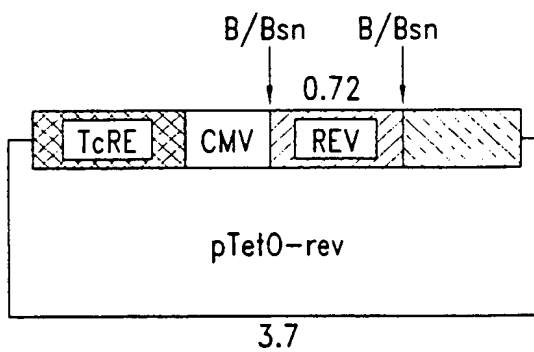
pCHGP-3

*Fig. 3C*

pCHGP-4

*Fig. 3D*

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*Fig. 4A**Fig. 4B*

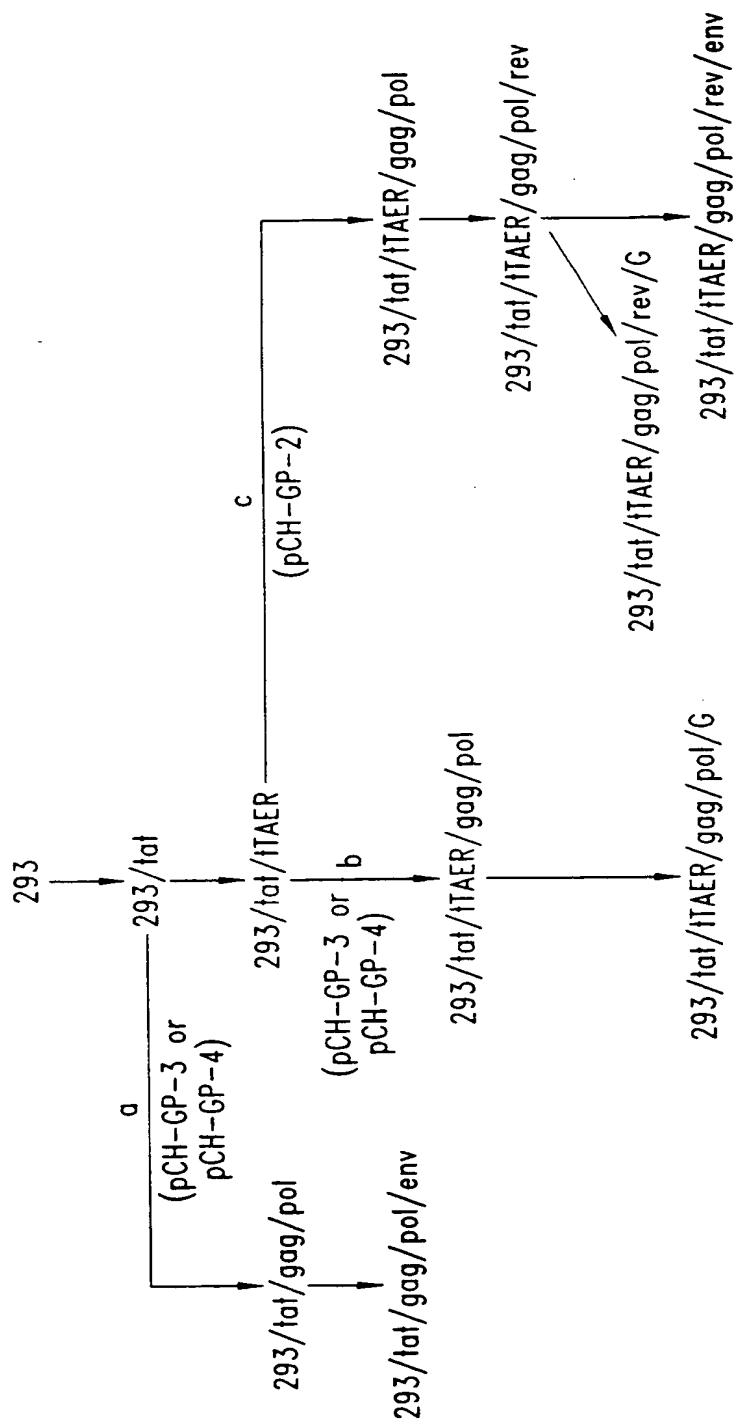


Fig. 5

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# LENTIVIRAL VECTORS

Comparison of the Genome Organizations of Leniti- and Oncoretroviruses

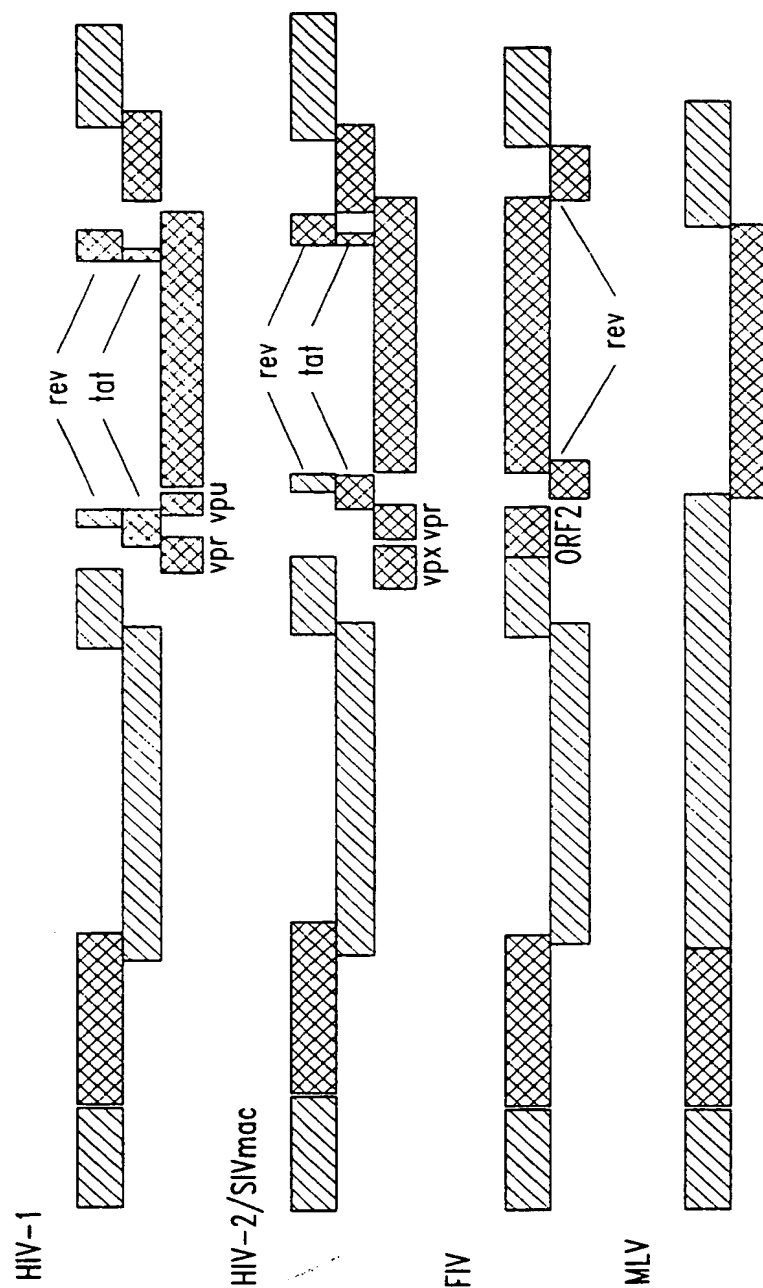


Fig. 6

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The effect of HIV-1 accessory proteins  
on vector production

CMV-HIV	Titer (IU/ml) <sup>a</sup>
Wild Type	$3.9 \times 10^6 \pm 6.8 \times 10^5$
vpr (-)	$8.3 \times 10^6 \pm 2.0 \times 10^6$
vpu (-)	$4.8 \times 10^6 \pm 9.9 \times 10^5$
vif (-)	$6.0 \times 10^6 \pm 6.5 \times 10^5$
nef (-)	$1.1 \times 10^7 \pm 1.0 \times 10^6$
nef (-); vpr (-)	$3.1 \times 10^6 \pm 3.1 \times 10^5$

<sup>a</sup>The vectors were harvested 24h after transfection and the titer was determined in HT1080 cells by counting of blue cells after X-Gal staining. The numbers are the average of triplicate experiments  $\pm$  standard deviation.

*Fig. 7*

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## Expression of p24 in pCHGP transfected cells

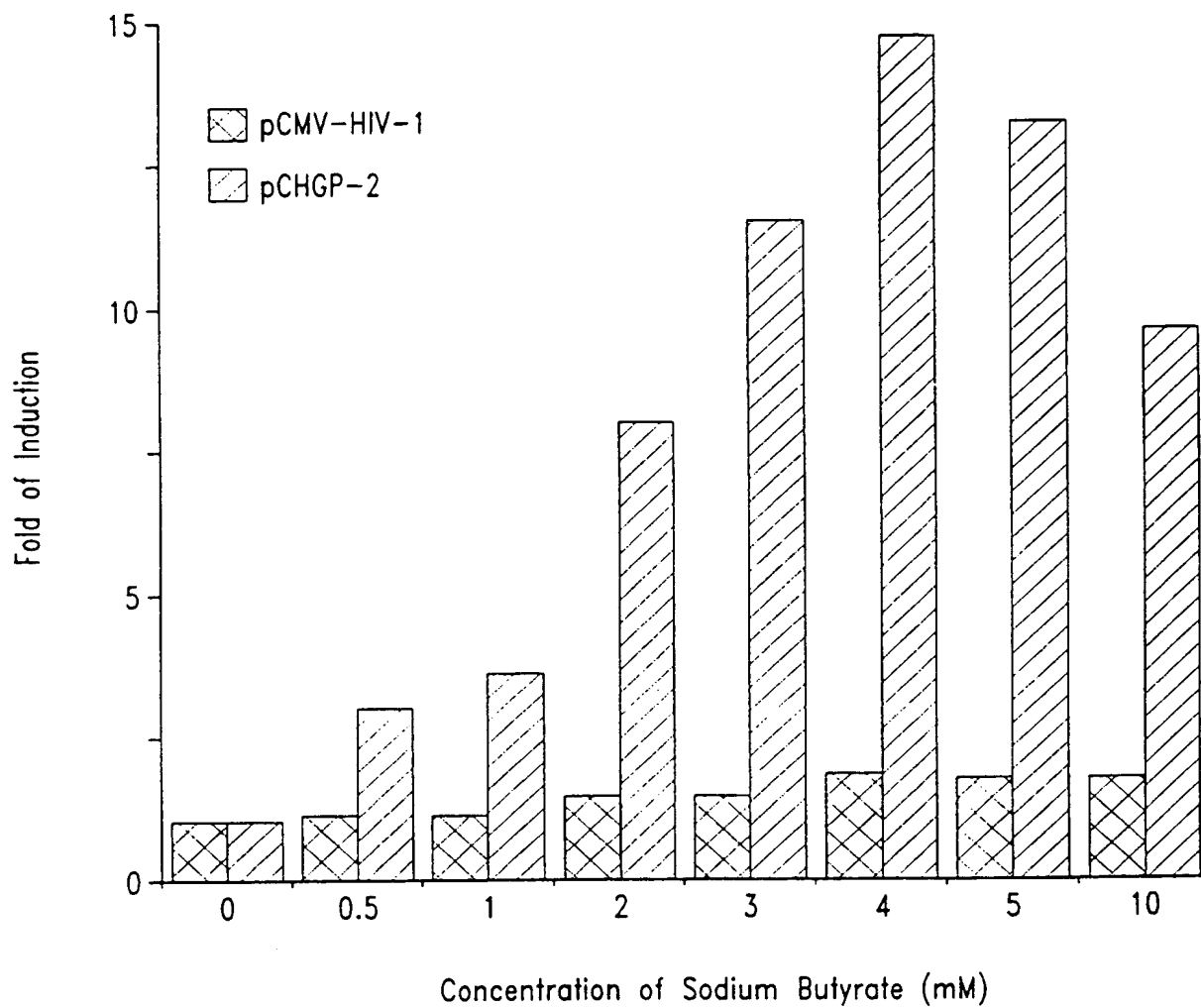
Plasmid Constructs	pCMV-Rev	p24 (ng/ml)
pCHGP-1	-	1.35 $\pm$ 0.17
	+	1.79 $\pm$ 0.14
pCHGP-2	-	3.52 $\pm$ 0.24
	+	98.84 $\pm$ 14.59
pCHGP-3	-	0.38 $\pm$ 0.09
	+	6.21 $\pm$ 0.65
pCHGP-4	-	10.21 $\pm$ 0.78
	+	16.41 $\pm$ 0.97
pCMV-HIV-1	-	6958 $\pm$ 349
	+	7471 $\pm$ 287

<sup>a</sup> 293T cells were transfected in a 10cm culture dish with 20  $\mu$ g of pCHGP as indicated and 10  $\mu$ g of either pCMV-Rev (+) or pBluescript (-). Forty-eight hours after transfection, the culture medium was assayed for p24 protein expression. The numbers are the average of 3 experiments  $\pm$  standard deviation.

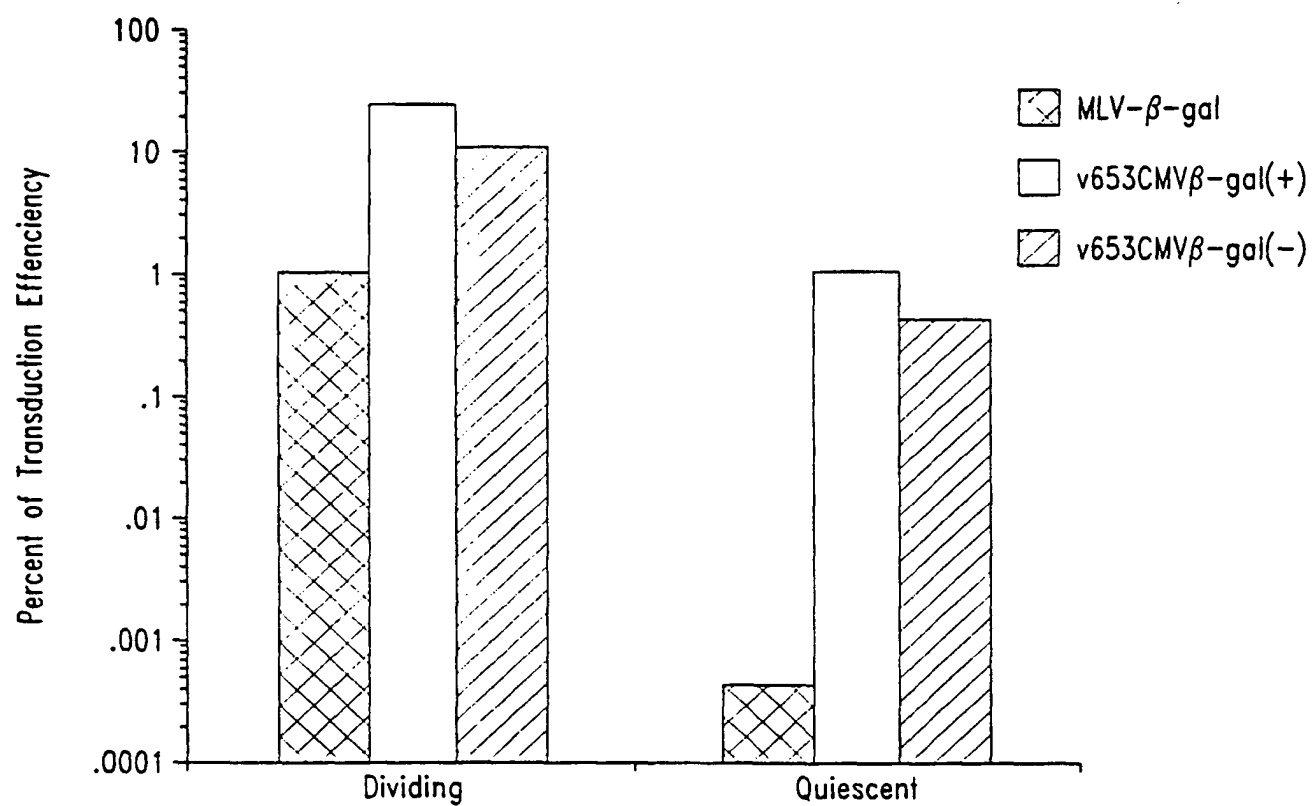
*Fig. 8*



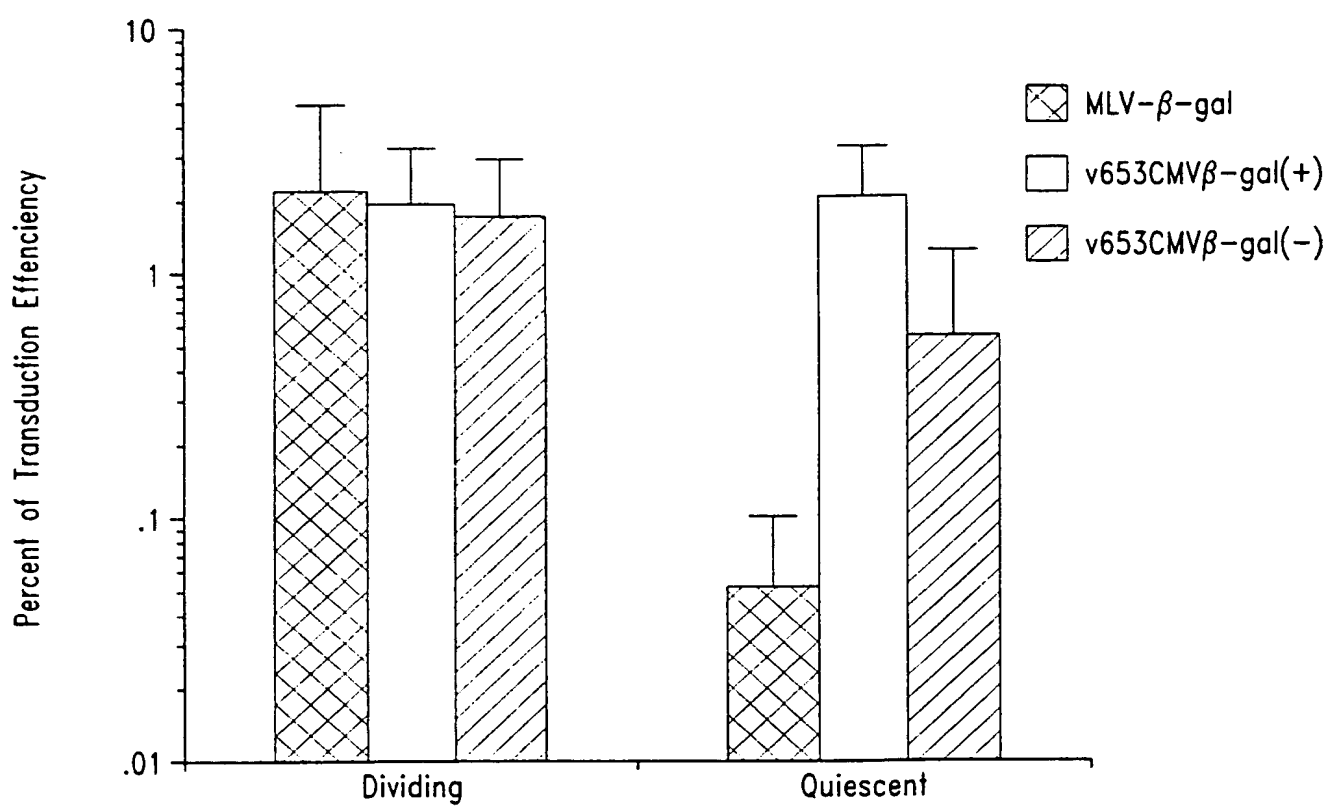
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*Fig. 9*

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*Fig. 10*

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*Fig. 11*





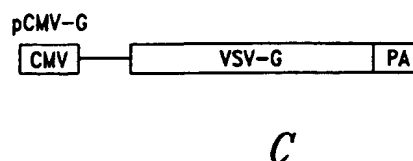
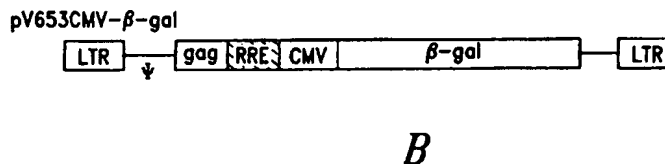
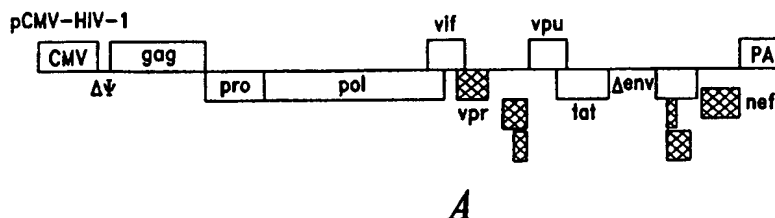
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/86, 5/10, 7/01, A61K 48/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 99/04026</b>
			(43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: <b>PCT/US98/14996</b>		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 20 July 1998 (20.07.98)		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 60/053,066 18 July 1997 (18.07.97) US			
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(74) Agents: MCMASTERS, David, D. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).		(88) Date of publication of the international search report: 8 April 1999 (08.04.99)	

## (54) Title: LENTIVIRAL VECTORS

## (57) Abstract

Lentiviral vectors are provided comprising a 5' lentiviral LTR, a tRNA binding site, a packaging signal, a promoter operably linked to one or more genes of interest, an origin of second strand DNA synthesis and a 3' lentiviral LTR, wherein the lentiviral vector contains a nuclear transport element that is not RRE. Also provided are expression cassettes and packaging cell lines for producing lentiviral vector particles.



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# INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 98/14996

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C12N15/86 C12N5/10 C12N7/01 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NALDINI L. ET AL.: "IN VIVO GENE DELIVERY AND STABLE TRANSDUCTION OF NONDIVIDING CELLS BY A LENTIVIRAL VECTOR" SCIENCE, vol. 272, no. 5259, 12 April 1996, pages 263-267, XP000583652	15.17, 20-24
Y	see the whole document	1-6,9
Y	BRAY M. ET AL.: "A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type I expression and replication Rev-independent." PROC. NATL. ACAD. SCI. USA, vol. 91, 1994 - 1256, page 1260 XP002092484 cited in the application see the whole document	1-6,9
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 12622 A (SALK INST FOR BIOLOGICAL STUDI) 10 April 1997	15.17, 20-24
Y	see the whole document	1-6.9
Y	--- HUANG J. AND LIANG T. J.: "A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products." MOLECULAR AND CELLULAR BIOLOGY, vol. 13, no. 12, 1993, pages 7476-7486, XP000676652 cited in the application see the whole document	1-6.9
X	--- US 5 585 263 A (HAMMARSKJOELD MARIE-LOUISE ET AL) 17 December 1996 see the whole document	10,14
X	--- WO 97 20052 A (CLINICAL TECHNOLOGIES INC) 5 June 1997 see page 9, last paragraph - page 10. paragraph 2	16,20-22
X	--- ORY D. S. ET AL.: "A STABLE HUMAN-DERIVED PACKAGING CELL LINE FOR PRODUCTION OF HIGH TITER RETROVIRUS/VESICULAR STOMATITIS VIRUS G PSEUDOTYPES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, October 1996, pages 11400-11406, XP002030784 see the whole document	17,20-24
A	--- BENDER M. A. ET AL.: "EVIDENCE THAT THE PACKAGING SIGNAL OF MOLONEY MURINE LEUKEMIA VIRUS EXTENDS INTO THE GAG REGION" JOURNAL OF VIROLOGY, vol. 61, no. 5, 19 May 1987, pages 1639-1646, XP002036903 see the whole document	3
A	--- MORGAN R. A. ET AL: "RETROVIRAL VECTORS CONTAINING PUTATIVE INTERNAL RIBOSOME ENTRY SITES: DEVELOPMENT OF A POLYCYSTRONIC GENE TRANSFER SYSTEM AND APPLICATIONS TO HUMAN GENE THERAPY" NUCLEIC ACIDS RESEARCH, vol. 20, no. 6, 1992, pages 1293-1299. XP002037514 see the whole document	7
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/14996

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LEVINE F. ET AL.: "Efficient gene expression in mammalian cells from a dicistronic transcriptional unit in an improved retroviral vector." GENE, vol. 108, 1991, pages 167-174, XP002092486 cited in the application see the whole document ---</p>	8
X	<p>WO 96 31602 A (ISIS INNOVATION ;KINGSMAN ALAN JOHN (GB)) 10 October 1996 see page 11, line 6 - line 15 ---</p>	28,29
P,X	<p>SRINIVASAKUMAR N. ET AL.: "The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type I vectors produced in stable packaging cell lines." JOURNAL OF VIROLOGY, vol. 71, no. 8, August 1997, pages 5841-5848, XP002092487 see the whole document -----</p>	1-3,5,9, 15-21,27

# INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 98/14996

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-27

A lentiviral vector; expression cassettes for gag/pol. tat, rev and VSV-G; host cells containing said expression cassettes; packaging cell lines; and a vector producing cell line.

2. Claims: 28,29

A method for enhancing production of infectious virus.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/US 98/14996

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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